A novel fluorescent receptor assay

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A Novel Fluorescent Receptor Assay Based Upon Receptors Embedded in Labeled Liposomes

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Preface

Receptors are (macro) molecules occurring in the cell membrane, in the cytoplasm (soluble receptors) of the cell, and in the cell nucleus, and are capable of binding a distinct chemical entity, after which the information present in that entity is transduced to the cell, thereby regulating a variety of biochemical processes necessary for the cell (and the organism) to function and survive. Both naturally occurring and synthetic molecules could serve as these chemical entities. Hormones, neurotransmitters and drugs are well-known examples of such (pharmacological active) receptor-binding substances.

For the qualitative and/or quantitative analysis of these substances, several methods are available, including separation methods, such as high performance liquid chromatography (HPLC). A special place among all methods available in biopharmaceutical analysis is taken by the receptor assay (RA). Receptor assays are, like immunoassays (IA), ligand-binding assays in which there is a competition between an analyte (e.g. the unknown quantity of drug in a blood- or urine sample) and a (labeled) ligand for binding to the same binding site, the receptor or antibody, respectively.

There are several advantages of RA over IA:

1. Substances belonging to the same pharmacological class can be determined, which includes active metabolites. Moreover, from enantiomeric substances, only the eutomer will bind to the receptor and, thus, can be detected. Cross-reactivity, one of the major problems encountered when antibodies are used, is not an issue in RA. Therefore, RA is a **selective** technique.

2. There is a direct correlation between pharmacological potency of a drug and the limit of detection (LOD). In other words, highly potent drugs that will be administered in low dosages, and thus are present in low concentrations in the biological matrix, can be detected easily because of their high affinity, and vice versa. Therefore, RA is a **sensitive** technique.
3. Receptors can be isolated relatively simply from animal tissue (e.g. brain tissue), which is often abundantly available from the slaughterhouse. In contrast, IA requires a complicated, costly production procedure because of the use of antibodies.

The binding of a ligand to its receptor can be detected by the use of a suitable label. Radioactive isotopes have become popular, due to the high sensitivity that can be obtained, and because the structure of the ligand will (in most cases) not be affected by the presence of the isotope (mainly ^{3}H).
However, the disadvantages of using radioactive materials are obvious. Therefore, alternative labels have been developed for the use in receptor assays. So far, the approach has been to use mainly fluorescent labeling of ligands for a variety of receptors. Since fluorophores generally are relatively large molecules, as a consequence, the labeling of the ligand in many cases causes a decrease in the binding affinity of the ligand for its receptor, due to e.g. steric hindrance.

To circumvent this problem, a different approach was used, as described in this thesis.
The basic idea is to label the receptor indirectly by incorporating the receptor protein in liposomes containing fluorescent functionalities. In order to obtain a competition assay, a ligand should be used allowing separation of protein liposomes which are either bound to the analyte or present in a different form (free or bound to the label).

For practical reasons and future automation it was decided to immobilize a ligand on the surface of wells of microtiter plates. For that purpose, the receptor has to be isolated from the (other) membrane (constituents) and reconstituted into liposomes. We used the benzodiazepine receptor as a model, because of our experience with fluorescent-ligand receptor assays for benzodiazepines. As a first step, we needed a suitable solubilization procedure to isolate the receptor from the brain membranes. Deoxycholic acid was chosen because of its ability to solubilize the benzodiazepine receptor in large amounts. Optimal incubation conditions were found by varying the concentration of detergent, the ionic strength and the pH of the incubation mixture.
Secondly, solubilized receptors had to be reconstituted in liposomes. Because exposure to detergents should be kept to a minimum, to avoid denaturation of the receptors, a fast procedure for the reconstitution was needed. Therefore, we preferred gelfiltration over dialysis. Several lipids and lipid combinations were used to obtain a proteoliposome suspension in which the benzodiazepine receptor displayed optimal binding characteristics with respect to affinity (measured as K_d) as well as the maximum amount of binding sites (B_{max}). Furthermore, the effect of freezing and thawing of the proteoliposome suspension on the binding parameters was examined, since freezing and thawing leads to an increase in the average size of the proteoliposomes, which might be favorable for the accommodation of large receptor proteins in the (phospho)lipid bilayers.

For the development of the fluorescent receptor assay, a ligand had to be immobilized to a solid phase. It is important that, after immobilization, the ligand retains its high affinity for the receptor. Didesethylflurazepam was chosen for the immobilization since it had been used previously for immobilization to agarose beads (with retention of high affinity) for affinity purification of the benzodiazepine receptor. The same protocol could be used

with only minor modifications. Maleic anhydride activated microtiter plates, normally used for the immobilization of proteins for e.g. immunoassays, were suitable for coupling of the ligand, because of the abundance of reactive groups.

The detection of the binding of the receptor-proteoliposomes to the immobilized ligands was accomplished by a modified radioreceptor assay, or by incorporating fluorescent lipids into the proteoliposomes. The use of fluorescent lipids has the advantage that many labels can be incorporated per single receptor molecule, thereby increasing the detection ability, and consequently the sensitivity of the actual assay. Other types of label were considered (e.g. chemiluminescent labels or enzymes), but these have the disadvantage that an additional step is required for detection.

For accurate quantitation of the analytes it is preferable to detect the bound fraction rather than the unbound fraction, since only a small proportion of the liposome population is likely to bind to the immobilized ligand. Besides, a relatively larger change in the amount of bound analyte can be expected, compared to the change in the free fraction, when analyte is present. Because it is expected that also proteoliposomes without receptors are present, a significant background signal will be codetermined in the free fraction.

List of Abbreviations

AAD	: adipic acid dihydrazide
Aso	: asolectin
B_{max}	: (maximum) number of binding sites present in the receptor material
Bodipy Fl C_5 HPC	: phosphatidyl choline labeled with Bodipy FL
BPL	: brain phospholipids
BSA	: bovine serum albumin
Cholh	: cholesteryl hemisuccinate
Dansyl DHPE	: phosphatidyl ethanolamine labeled with dansyl chloride
EDTA	: ethylenediaminetetraacetic acid
EPC	: egg phosphatidyl choline
Fluorescein DHPE	: phosphatidyl ethanolamine labeled with fluorescein
FNM	: flunitrazepam
FRA	: fluorescent receptor assay
GABA	: γ-aminobutyric acid
IA	: immunoassays
IC_{50}	: concentration inhibitor that displaces 50% of the bound labeled ligand
K_d	: equilibrium dissociation constant
K_i	: inhibition constant
MW	: molecular weight

PC	: phosphatidyl choline
PE	: phosphatidyl ethanolamine
PEG	: polyethylene glycol
RA	: receptor assays
RIA	: radioimmunoassays
Ro7-1986	: didesethylflurazepam
RRA	: radioreceptor assays
Tris	: tris(hydroxymethyl)aminomethane

Chapter 1

Introduction

1.1 Methods in Biopharmaceutical Analysis

In order to qualitatively or quantitatively determine an analyte, several methods are available in biopharmaceutical analysis. These methods include (a) direct methods such as spectrophotometry or polarography, (b) separation methods including high performance liquid chromatography (HPLC), gas chromatography (GC), (capillary) electrophoresis and thin layer chromatography (TLC) and (c) ligand binding assays like immunoassays and receptor assays.

The discovery that pharmacologically active substances such as hormones, neurotransmitters and drugs exert their action through specific receptor sites led to the development of the receptor assay.
Receptor assays are unique amongst other methods used in bioanalysis, since they do not only determine the concentration of the analyte, but are also capable of measuring the affinity of the analyte to the specific receptor site. Receptor assays utilize the first step (binding) in a cascade of physiological events (Fig. 1.1).

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1.2 Receptor Biochemistry

By definition, a receptor is a (macro) molecule which is able to *recognize* a distinct chemical entity amongst many others, and which *transduces* the information contained within that entity to the cell, resulting eventually in a response such as activation of an enzyme (e.g. adenylyl cyclase), alteration of membrane permeability etc. Most receptors are transmembrane glycoproteins naturally occurring in cell membranes of different tissues and organs, although there are also soluble receptors found in the cytoplasm and the nucleus of the cell (e.g. receptors for steroids and thyroid hormones). In this chapter only membrane bound receptors will be described; however, the theory of receptor binding is also valid for soluble receptors. (Membrane) receptors can be classified according to pharmacological or structural characteristics.

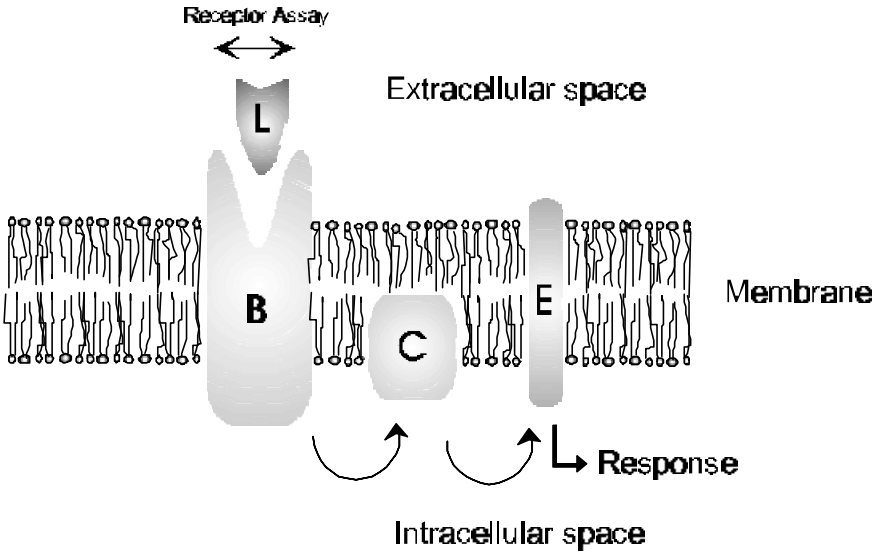

Figure 1.1 Cascade of biological events that take place in a cell when a bioactive substance binds to the binding site of a receptor. Receptor assays only make use of the first (binding) step. L = bioactive compound (ligand); B = binding site (receptor); C = coupling component (e.g. G-protein); E = effector (e.g. enzyme or ion channel).

Pharmacologically, receptors are classified by their (relative) affinity and selectivity of (1) antagonists (analytes with affinity for the receptor, but no efficacy) and (2) agonists (analytes with both affinity and efficacy) as defined by Kenakin *et al.* (1992). For receptor classification purposes, antagonists are preferably used. Requirements are that measurements should be made under equilibrium conditions and that the binding is competitive.

Structural (and functional) analysis distinguishes three main receptor groups:

(a) Receptors with a single transmembrane segment (e.g. the epidermal growth factor receptor (EGFR) and the insulin receptor (IR)).

(b) Hetero-oligomeric receptors containing one or multiple binding sites and an ion channel (e.g. the $GABA_A$ receptor, Fischer and Olsen 1986).

(c) Receptors interacting with GTP-binding proteins (G-proteins) usually containing seven hydrophobic transmembrane segments; e.g. the muscarinic acetylcholine receptor (mACR; Kerlavage *et al.*, 1987) and the β-adrenergic receptor (β-AR; Lefkowitz *et al.*, 1983).

Many receptors are built from more than one subunit, for which multiple subtypes can exist. For example, for the $GABA_A$ receptor, several subunits have been discovered including α(1-6), β(1-4), γ(1-3), δ and ρ(1,2) subtypes (Levitan *et al.*, 1988 and Macdonald and Angelotti 1993). Various receptors have been cloned and sequenced in recent years, as described for the muscarinic acetylcholine receptor (Kubo *et al.*, 1986), the epidermal growth factor receptor (Ullrich *et al.*, 1984) and the $GABA_A$ receptor (Macdonald and Angelotti 1993). Cloning of receptors (or receptor subunits) followed by selective expression in carcinoma cells or oocytes has given a better insight in the molecular basis of receptor functionality and what subtypes might be needed for binding of analytes. This may eventually lead to a more complete understanding of the basis of

neurotransmitter and hormone action, the role of receptors in disease and health, and might be advantageous in future drug development programs.

1.3 Ligand Binding Assays

1.3.1 The Immunoassay

In 1959, Yalow and Berson published their famous article about quantitation of insulin in serum, using ^{125}I-insulin and anti-insulin antibodies as binding proteins. The basic idea behind this method was that there would be a competition between a labeled antigen and an unlabeled antigen for the same (limited amount of) antigen binding sites. Separation of the bound and free fractions made it possible to determine the amount of labeled antigen in either one of the fractions. This principle is still valid for the numerous variations made on the assay since then.

The interaction between a binding protein and a ligand is, in the case of an immunoassay, a selective antigen-antibody interaction. In all types of immunoassays, be it radio-immunoassays, fluorescence immunoassays or enzyme-immunoassays, there is a distinction between competitive and non-competitive methods. Detailed information on experimental set-ups and theoretical backgrounds can be found in the excellent antibody manual by Harlow and Lane (1988).

Competitive immunoassays

In this type of assay there is competition between a known amount of labeled antigen and a sample (an unknown amount of unlabeled antigen) or standard, for a limited concentration of antibodies. An increase in antigen in the sample will lower the amount of labeled antigen that can bind to the antibodies. Many of today's immunoassays are heterogeneous; i.e. the free and bound antigen fractions must be separated. Several separation methods are available, including precipitation of the bound fraction with a secondary antibody and specific coating

of a solid phase with the antigen or antibody, followed (after the incubation) by a simple washing step to remove the unbound fraction. The amount of label in either one of the fractions, for practical reasons this is usually the bound fraction, can be determined, and is a measure for the concentration of antigen in the sample. As an alternative, labeled antibodies can be used. A commonly used modification on the procedure described above is the so-called 'sequential saturation' or titration method, in which the (unknown) amount of antigen in the sample (or standard) is bound to a known concentration of excess antibody. As a second step, the free antibody binding sites will be occupied by labeled antigen.

Non-competitive immunoassays

Also several non-competitive immunoassays exist, e.g. direct or indirect two-site (sandwich) immunoassays, and direct or indirect one-site immunoassays. In the direct two-site assay, an excess amount of antibodies (Al_1) bound to a solid phase are incubated with standards or a sample, resulting in binding of the unknown amount of antigen to the immobilized antibody. After washing, an excess of labeled antibody (Al_2^*) is added which will bind to the already fixed antigen. After separation of bound and free fractions the activity in the bound fraction can be determined. A disadvantage of this method is that it can only be used when the antigen is large enough to accommodate two antibodies. The indirect method is similar to the direct one, except that the secondary antibodies (Al_2) are unlabeled. Here, a third labeled antibody directed against immunoglobulins is added for detection.

In the direct one-site immunoassay, the sample is incubated with an excess labeled antibody (Al_1^*) followed by incubation with an excess antigen immobilized on a solid phase. In this case the non-bound antibodies will bind to the solid phase, and binding will decrease with increasing amounts of antigen in the sample. The indirect one-site immunoassay makes use of a third, labeled, antibody, as in the two-site method.

1.3.2 The Receptor Assay

Principles of the receptor assay

The first example of a quantitative receptor assay was published by Lefkowitz *et al.* (1970) describing the competition between ACTH and ^{125}I-ACTH for specific receptor sites in homogenates of adrenal cortical tissue. The same principles originally used for immunoassays are applicable for receptor assays. In both immunoassays and receptor assays the analyte [L] competes with a labeled ligand [L^*] for the same binding site on the protein [antibody [A] or receptor [R], respectively] as shown for the receptor assay in equation (1).

$$[R] + [L] + [L^*] \leftrightarrow [RL] + [RL^*] \qquad (1)$$

Depending on the affinity of the analyte for its receptor, very high sensitivity can be achieved. It is not uncommon that nanograms or even picograms of analyte can be measured. Another feature of a receptor assay is the selectivity. Most receptors bind a distinct class of structurally related molecules. This means that only the total of (pharmacologically) active ligand and metabolites can be detected by the assay when these molecules bind to the same site. This is also a limitation since no discrimination can be made whether the detected molecule is the parent compound or one of its active metabolites. Cross reactivity with structurally related but pharmacologically inactive molecules (a major drawback in immunoassays) does not occur. Also, when chiral substances are involved, only the active enantiomer (eutomer) will bind to the receptor whereas the inactive distomer will not. Radioreceptor assays have been described for a large number of receptor classes, including benzodiazepine (Hunt *et al.*, 1978, Lund 1981), muscarinic (Ensing *et al.*, 1984 and 1988), cholinergic (Covarrubias *et al.*, 1986), alpha-adrenergic (Greenberg *et al.*, 1976), beta-adrenergic (Innis *et al.*, 1978, Iwo *et al.*, 1985), opiate (Villiger *et al.*, 1981) receptors as well as for human growth hormone

(Ilondo *et al.*, 1991), tricyclic anti-depressants (Innis *et al.*, 1979) and neuroleptics (Creese and Snyder 1977).

Receptor-ligand binding theory

The relationship between a fixed amount of receptor, labeled ligand (e.g. radioligand) and the formed complex is shown in equation (2), where [R], [L^*] and [RL^*] are the concentrations of receptor, labeled ligand and complex, respectively, and k_{-1} and k_1 are the dissociation and association rate constants, respectively:

$$[R] + [L^*] \leftrightarrow [RL^*] \qquad (2)$$

Since the amount of receptors is limited, saturation of the binding sites will occur at high concentrations of labeled drug. Figure 1.2 shows a typical saturation curve for benzodiazepine receptors by [^{3}H]flunitrazepam. The total amount of binding sites (B_{max}) is found on the ordinate at the point where the curve reaches its plateau. The amount of ligand that gives a 50% saturation of the receptor represents the dissociation constant K_d.

B_{max} and K_d can also be calculated using equation (3):

$$[RL^*] = \frac{[L^*] * B_{max}}{[L^*] + K_d} \qquad (3)$$

The dissociation constant K_d is inversely proportional to the affinity of the analyte (or ligand) for the receptor and is defined by the ratio of the dissociation and association rate constants:

$$K_d = k_{-1} / k_1 \qquad (4)$$

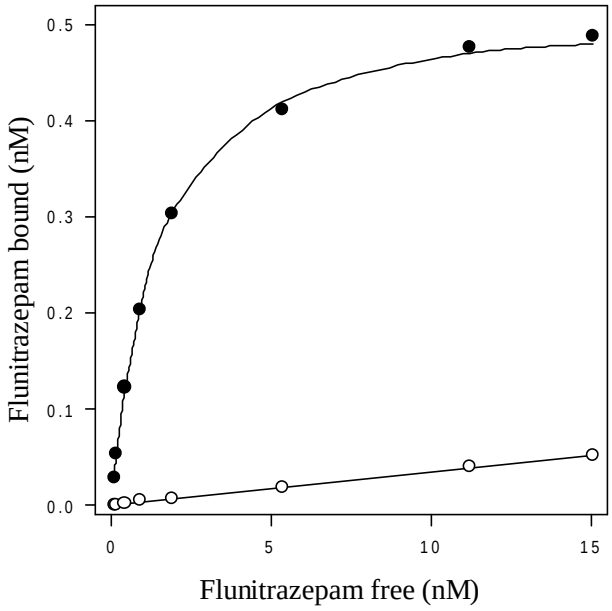

Figure 1.2 Typical saturation curve for the membrane-bound benzodiazepine receptor using [^{3}H]flunitrazepam as the labeled ligand. Closed circles represent specific binding and open circles the nonspecific binding. Binding parameters (K_d and B_{max}) can be calculated using the EBDA-LIGAND computer fitting program (Version 4.0; Biosoft, Cambridge, UK), based upon a program originally developed by Munson and Rodbard (1980), considering a one-site binding model.

Addition of a competitive (unlabeled) analyte will displace a certain amount of labeled ligand, depending on the concentration of the former and on its equilibrium binding constant K_d, resulting in two types of receptor complexes, as described in equation (1). By varying the amount of analyte and keeping the concentration of labeled ligand and receptor constant, calibration curves can be constructed, as shown in figure 1.3. The IC_{50} value, i.e. the amount of analyte displacing 50% of the bound labeled ligand can be determined from these curves.

The affinity constant of the analyte (K_i) is related to the IC_{50} as described by the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$IC_{50} = K_i * (1 + \frac{[L^*]}{K_d} \quad (5)$$

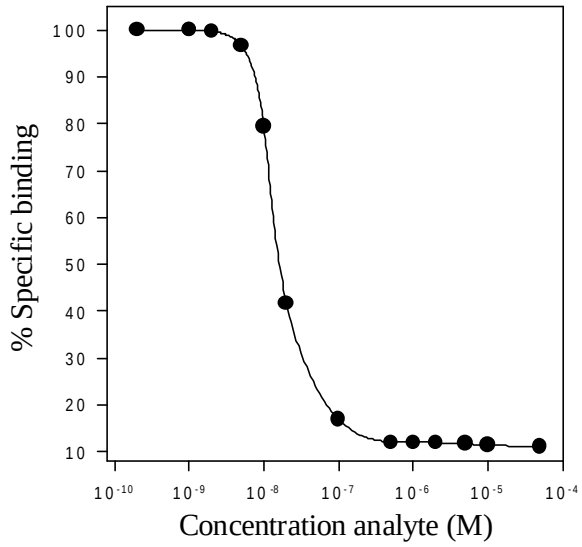

Figure 1.3 Hypothetical calibration curve of an analyte displacing a labeled ligand from its receptor.

Sensitivity of receptor assays

The sensitivity of specific binding of a ligand to its receptor depends on the ratio of concentration/K_d of the ligand being assayed.

An important parameter is the limit of detection (LOD), which can be defined as the minimum concentration of analyte at which the fraction of bound labeled ligand is significantly smaller than the fraction of bound labeled ligand in the absence of analyte, and can be calculated using equation (6):

$$\mathrm{LOD} = \gamma_1[1 + \frac{K_n^* * K_d^*}{[R]_0}(1 + \frac{[L^*]}{K_d^*})](1 + \frac{[L^*]}{K_d^*})K_d \qquad (6)$$

where:

γ_1 = a parameter from Student *t*-test which characterizes the error of the determination of the concentration of labeled ligand in the absence of analyte

K_n^* = the constant for non-specific binding of the labeled ligand

K_d^* = the dissociation constant of the labeled ligand

$[R]_0$ = the total receptor concentration

$[L^*]$ = the concentration of the free labeled ligand

K_d = the dissociation constant of the analyte

It can be seen from equation (6) that several factors determine the sensitivity of a receptor assay.

(a) The LOD is directly proportional to the K_d of the analyte. In other words, when the analyte has a high affinity (low K_d), less of it can be detected.

(b) Since the LOD is also directly proportional to the concentration of labeled ligand, a minimum of the labeled ligand should be used to increase sensitivity. A free concentration of labeled ligand equal or close to the dissociation constant is considered to be a good compromise.

(c) The lower the amount of non-specific binding, the higher the sensitivity, resulting in a decrease in the LOD.

Selectivity of receptor assays

During a receptor assay only pharmacologically active compounds will bind to its receptor, while inactive compounds belonging to the same structural class or compounds belonging to another structural class will not bind. Selectivity is determined by the nature of the receptor preparation, the type of labeled ligand used in the assay and the binding properties of the analyte being assayed. Receptor

preparations containing a single receptor or a heterogeneous population of binding sites can be used. If a heterogeneous receptor population is used, it is important to choose the (labeled) ligand with care. If the labeled ligand and the analyte bind to more than one class of receptors, depletion of both ligand and analyte might occur when using heterogeneous receptors. For example, [^{3}H]-spiperone is a potential radioligand for D_2 dopamine receptors, but is also labeling $5HT_2$ serotonin receptors, α_1-adrenergic receptors and the spirodecanone acceptor (Strange 1987). Specific brain regions (e.g. corpus striatum) containing larger amounts of D_2 receptors may be used to minimize the influence of binding to other receptors.

A more complicated situation occurs when a single receptor binds more than one class of ligands. For example, the prolactin receptor is closely related to the growth hormone receptor (Boutin *et al.*, 1988) and will bind human prolactin and human growth hormone with nearly equal affinity. However, the use of cultured human lymphocytes containing specific growth hormone receptors (Lesniak *et al.*, 1974 and 1977) can circumvent this problem.

1.3.3 Methodology of the Receptor Assay

Receptor preparations

The receptor preparations needed for receptor assays are usually easily obtainable from animal tissue, such as brain, liver or heart. Receptor density and the pharmacological characteristics are among the most important factors in selecting the most suitable receptor source. Besides the receptor of interest, other receptors and non-receptor proteins will be found in the membranes, often causing substantial non-specific binding. Receptor densities are typically in the range of fmol-pmol per mg of total protein. A crude membrane homogenate prepared by homogenizing animal tissue or organ in a 0.32 *M* sucrose solution will be

satisfactory for most purposes. Differential centrifugation is required to obtain a more purified subcellular fraction. Further purification is laborious and only necessary when the receptor density is extremely low, or when the receptor protein itself has to be characterized on a molecular level. A general approach for the purification of receptors is to solubilize the membranes with an appropriate detergent (see Section 1.4), followed by isolation of the receptor protein on e.g. an affinity gel using immobilized ligand (T. Haga *et al.*, 1990). A 100- to 1000-fold purification may be achieved in this way. However, the method may also be combined with other procedures, such as ion-exchange, hydroxyapetite or lectin column chromatography. Aducci *et al.*, (1993) has achieved a 5000-fold purification of the fusicoccin receptor from maize using a combination of adsorption, ion-exchange and gel-filtration chromatographies. On the other hand, when trying to purify or solubilize membrane proteins, care must be taken to prevent denaturation of the proteins.

As an alternative, recombinant receptors expressed by a single cell line may be used. The advantage of using this type of receptor preparation is that identical receptors can be produced in larger quantities. Besides, non-specific binding to irrelevant proteins will be significantly reduced.

The choice of labeled ligand

Most labeled ligands used in the past three decades were radioligands. The main advantage of using radioligands (mainly tritium) in receptor assays is that the spatial structure of the ligand is not or only slightly modified by the incorporation of an isotope. Therefore, the affinity of the labeled ligand can be expected to be similar to the unlabeled compound. Besides, they are chemically stable and can be sensitively detected over a wide range. However, there are several disadvantages in using radiolabels as well, including high costs, radioactive waste, health hazards, and the need of special equipment and licenses. For this reason fluorescent labels for the use in receptor assays have been developed, as described

for the α- and β-adrenergic and the opiate receptor by Corrêa *et al.*, (1980), the benzodiazepine receptor (Takeuchi and Rechnitz, 1991a, and Janssen, 1997) and the estrogen receptor (Hwang *et al.*, 1992). However, the attachment of a (bulky) fluorescent moiety to a ligand often results in diminished affinity of the ligand to its receptor. Moreover, since ligands are bound to distinct entities of the of the receptor protein, many positions on the ligand are unsuitable for the attachment of fluorescent labels.

Biotin labeled benzodiazepines have been used in an avidin-biotin binding assay by Takeuchi *et al.*, (1991b), and Hallowell and Rechnitz (1987) used phenylcyclidine coupled with glucose-6-phosphate dehydrogenase in an enzyme-amplified receptor assay for the acetylcholine receptor. A promising development is the use of chemiluminescent labels. The latter have been used in a number of immunoassays with similar or higher sensitivity than radioactive probes as described by Weeks *et al.*, (1983) and Stabler and Siegel (1990). Katoh *et al.*, (1995) used acridinium labeled fibrinogen in a chemiluminescent receptor assay for the exposed platelet fibrinogen receptor and found similar K_I values as compared with ^{125}I-labeled fibrinogen.

Some general criteria, which apply to all labeled ligands used, are:

(a) They should bind to the same receptor site as the unlabeled ligands.

(b) They should be chemically stable and resistant to enzymatic and hydrolytic degradation. The latter might be of particular concern when peptides are used as labeled ligands.

(c) Non-specific binding to non-receptor sites and materials used must be minimal.

(d) They should preferably be eutomers, i.e. the pharmacologically most potent enantiomers.

Radioligands have to meet some additional criteria:

(e) They should be of high specific activity. This is necessary because the receptor density in most tissues and organs is limited.

(f) They have to be radiochemically pure. Labeled impurities can contribute to artifacts, such as curvilinear Scatchard plots, Hill coefficients smaller than unity and poor specific to non-specific binding ratios.

Based on the discovery that many receptors can be subdivided into subtypes, it should be clear that the labeled ligand and analyte should select the same receptor subtype in order to obtain maximal specificity and sensitivity. Moreover, differences in hydrophilic or lipophilic properties of ligands may also contribute to heterogeneity in binding.

Separation of bound and free labeled ligand

During a receptor assay the labeled ligand and analyte are incubated under well-controlled conditions. The incubation temperature and time are dependent on the kinetics (association and dissociation rates of the ligand and analyte), but is typically 30 to 60 minutes at 0°C to 37°C. In order to measure the amount of labeled ligand bound to its receptor, separation of the bound and free fraction is necessary. Separation methods include filtration through glass fiber filters or centrifugation for membrane bound receptors, and dialysis, gel filtration, precipitation of the receptor-ligand complex with PEG 6000-8000 or ammonium sulfate followed by centrifugation or filtration, or adsorption of free ligand followed by centrifugation when using soluble or solubilized receptors.

Centrifugation and filtration methods have the advantage that both bound and free fraction can be collected and measured separately. Filtration, gel filtration and ligand absorption assays are principally non-equilibrium techniques, causing ligand dissociation, and are therefore not suitable for rapidly dissociating ligands. Equilibrium dialysis and gel filtration have the disadvantage of being rather time

consuming, which make them not practical when many samples have to be analyzed.

1.4 Solubilization of Membrane Receptors

Solubilization of biological membranes by detergents eventually results in the separation of the individual components (proteins and lipids) which the membrane is built of, and is the necessary first step in the purification of membrane proteins. Detergents are, like phospholipids, amphiphilic molecules, i.e. they have a hydrophilic 'head' and a hydrophobic 'tail'. Many detergents are commercially available, including non-ionic detergents such as the Tritons (e.g. Triton X-100 and Triton X-405), Nonidet P-40, Lubrol PX, octyl glucoside and octyl thioglucoside and digitonin. A second class of detergents are the ionic detergents of which the anionic detergents sodium cholate and sodium deoxycholate are the most frequently used. Finally, zwitterionic detergents such as CHAPS and CHAPSO may be used. The way a detergent solubilizes the membrane depends highly on the type of detergent used as well as on the properties of the membrane. Moreover, the absolute and relative concentrations of the detergent, phospholipid and proteins present during the solubilization play a key role in the solubilizing potency, i.e. final concentration of the detergent used.

Addition of a small amount of detergent to a membrane suspension (below the critical micellar concentration, CMC, which is the concentration at which the detergent molecules form micelles) will result in partitioning of the detergent molecule between the aqueous phase and the membrane. Increasing the detergent concentration will result in an increase in concentration in the bilayer, eventually causing fragmentation of the membrane. When the detergent concentration increases above the CMC, micelles will form, resulting in large-scale solubilization of membrane proteins and lipids. Both mixed detergent-lipid-protein micelles and detergent-lipid micelles can be found at this stage. A further increase

in detergent concentration will mainly result in an increase of detergent micelles and might also result in protein inactivation or even protein aggregation due to the depletion of essential endogenous lipids.

Designing a solubilization procedure is mainly a case of trial and error, although some rational considerations often can be made. For instance, the receptor-ion channel complexes, e.g. the $GABA_A$-benzodiazepine receptor, can be solubilized effectively by ionic detergents such as sodium cholate or deoxycholate, although also non-ionic and zwitterionic detergents have been used with some success. Typically, different membrane proteins will become soluble at different detergent concentrations, while some proteins, e.g. those membrane proteins attached to the cytoskeleton, may not be solubilized at all.

1.5 Liposomes

Liposomes are spherical vesicles consisting of one or more lipid bilayers (usually composed of phospholipids) surrounding an aqueous inner volume and are formed spontaneously when lipid molecules are dispersed in aqueous media. Liposomes are often used as a model membrane system, since they can be prepared in such a way that they are similar to the native membrane bilayer. They were first described by Bangham *et al.* (1965) who studied the nature of cell membranes. Today, most interest into applications of liposomes is in the field of drug delivery (Crommelin *et al.*, 1997 and Siler-Marinkovic *et al.*, 1997), gene therapy (Smith *et al.*, 1993) and liposomes are even produced by the cosmetic industry as constituents of creams and lotions. As an analytical tool, liposomes have been immobilized in gel beads for the chromatographical analysis of the specific interactions between solutes and membrane proteins (Lundahl and Yang, 1991) or drug partitioning in lipid bilayers (Beigi *et al.*, 1995). Liposomes are also used in immunoassays (Axelsson *et al.*, 1981 and Litzinger and Huang, 1992) by cross-linking antigens or antibodies to the liposome bilayer. Most types of

immunoassays can be used in combination with liposomes. In some cases the main difference is the replacement of the enzyme-substrate reaction used in the 'traditional' assay, by liposomes containing encapsulated marker molecules such as fluorophores, chromophores or chemiluminescent molecules. Membrane proteins can be purified and reconstituted in liposomes in order to study their structure and function (see Chapter 3).

Structure of liposomes

The (most) commonly used structural components of liposomes are phospholipids (Figure 1.4), of which the most common are phosphatidylcholines (lecithin).

Phosphatidylcholines are primarily used in liposomes because of their low cost and neutral charge. Naturally occurring phosphatidylcholines (and other phosphatidyl glycero-phospholipids) are mixtures of phospholipids with different chain lengths and varying degrees of unsaturation. Other neutral phospholipids are sphingomyelin and phosphatidylethanolamine. Negatively charged phospholipids include phosphatidylglycerol, phosphatidic acid and phosphatidylserine.

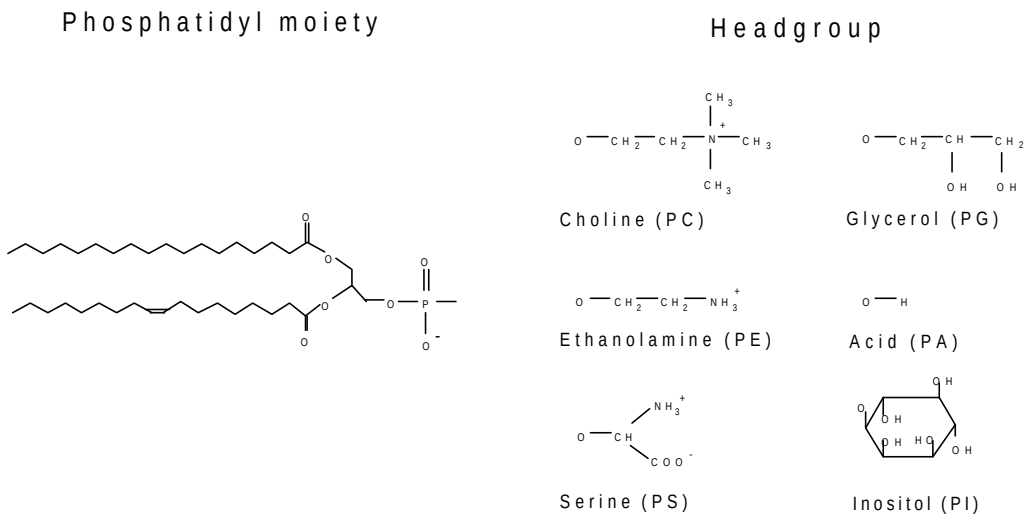

Figure 1.4 Some commonly occurring phosphatidyl glycero-phospholipids. PC and PE are neutral since the positively charged nitrogen in the choline and ethanolamine headgroups neutralizes the negative charge on the phosphate. All other (phosphatidyl) glycero-phospholipids are negatively charged, as PA can even have a double negative charge.

Cholesterol is another important constituent of biological membranes, since it plays a key role in controlling the fluidity and permeability of the membrane.

Preparation of liposomes should be simple and reproducible, with sizes as homogeneous as possible. Several types of liposomes can be distinguished, ranging from small unilamellar vesicles (SUV) with diameters below 100 nm, to large unilamellar vesicles (LUV) with sizes between 100 nm and 1000 nm. Multilamellar vesicles (MLV) are comparable in size to LUVs and are generally consisting of at least five concentric lamellae.

When size-exclusion chromatography (better known as gel filtration) is used as the method for detergent depletion, the formed (proteo)liposomes are rather small

(100-200 nm on average) and mainly unilamellar. Since the method is simple and fast, it is frequently used in reconstitution studies.

1.6 Concluding Remarks

Receptor assays offer the possibility to determine the pharmacological properties of hormones, neurotransmitters and drugs. Moreover, receptor assays can be used for selective and sensitive determination of these compounds in biological and other relevant matrices. In this regard, it should be noted that receptor assays are not mass detectors, but that their principle is based on affinity. Thus, very potent substances (e.g. drugs) that are given in low dosages and are present in minute amounts in the body, can often be analyzed properly with receptor assays. Also, since the technique is simple and rapid it may be particularly useful to screen for the presence of representatives from a certain pharmacological class (opiates, benzodiazepines, neuroleptics, etc.), e.g. in clinical and forensic toxicology.
The labeled ligand, the receptor preparation and the incubation conditions have to be chosen carefully in order to obtain a stable and, in most cases, near-physiological medium. Association and dissociation rate constants of the ligand and analyte determine the incubation time and the method needed to separate the bound and free fractions. Limitations of receptor assays, caused mainly by the type of biological materials used, can be reduced by using standardized receptor preparations (e.g. purified or cloned receptors can lower the amount of non-specific binding and hence increase the sensitivity of the assay) and by automation of the procedure. The development of non-radioactive labeled ligands, and non-radioactive receptor assays in general, will make the assay more accessible for use in routine laboratories.

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Chapter 2

Solubilization of the Bovine Brain Benzodiazepine Receptor

2.1 Introduction

To study the structure and function of membrane-bound receptors (and membrane proteins in general) it is often necessary to solubilize the proteins, in order to separate them from their lipid environment, followed by further purification using e.g. affinity chromatographical techniques. Moreover, solubilization of membrane proteins is also the first step in the reconstitution of those proteins (see also Chapter 3).

When radioreceptor assays (RRA) are used to study the interaction of a ligand (drug, hormone or neurotransmitter) with its receptor protein, the use of a crude membrane homogenate as the receptor source is usually sufficient. Solubilized receptors may be needed when non-radioactive receptor assays are used. For example, in developing a fluorescent receptor assay (FRA) for the benzodiazepine receptor, the presence of membrane-bound benzodiazepine receptors caused a high background signal, due to scattering and autofluorescence, which was minimized when a solubilized receptor preparation was used (Janssen *et al.*, 1996).

Modified version of:
Viel, G.T., Yang, Q., Lundahl, P., Ensing, K. and de Zeeuw, R.A. (1997) Size-exclusion chromatographic reconstitution of the bovine brain benzodiazepine receptor. Effects of lipid environment on the binding characteristics. *J. Chromatogr. A* **776**, 101-107.

The major inhibitory neurotransmitter in the mammalian central nervous system, γ-aminobutyric acid (GABA), interacts with central and peripheral receptor subtypes. GABA induces membrane hyperpolarization by opening a chloride channel in the $GABA_A$ receptor, which is part of a multireceptor protein, also containing binding sites for psychoactive drugs such as the 1,4-benzodiazepines, the barbiturates and for convulsant compounds like picrotoxinin.

The large multireceptor protein ($M_r \approx 300\ 000$) can be solubilized by a variety of detergents, such as Triton X-100 (Lang *et al.*, 1979), 1-O-*n*-octyl β-D-glucopyranoside (β-octyl glucoside) (Hammond and Martin, 1986), sodium cholate and sodium deoxycholate (Asano and Ogasawara, 1980 and Asano and Ogasawara, 1981) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (Stephenson and Olsen, 1982). Though CHAPS appears to be the only detergent capable of solubilizing the $GABA_A$ receptor with retention of all types of binding sites (Hammond and Martin, 1986 and Stephenson and Olsen, 1982), deoxycholate solubilizes larger amounts of GABA and benzodiazepine sites than CHAPS (Hammond and Martin, 1986; Asano and Ogasawara, 1980; Asano and Ogasawara, 1981 and Stephenson and Olsen, 1982).

This chapter describes the solubilization of the benzodiazepine receptor with deoxycholate. Saturation binding experiments were done to examine the binding characteristics of the solubilized receptor preparation and were compared to values found with membrane-bound receptors. The effect of deoxycholate on the solubilized receptor binding assay was studied, as well as

the effects of the pH of the incubation medium and the presence or absence of KCl during the solubilization procedure.

2.2 Materials and Methods

Materials

Bovine serum albumin (type 5, 96-99%), bovine globulins (Cohn fractions II and III), benzamidine hydrochloride (97%), bacitracin (50,000 units/g) and sodium deoxycholate (>95%) were bought from Sigma (St. Louis, MO, USA). [*N-methyl*-^{3}H]flunitrazepam (82.0 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Lorazepam was kindly provided by Wyeth Laboratoria (Hoofddorp, The Netherlands). Other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Polyethylene glycol (PEG) 6000 was supplied by Genfarma (Maarssen, The Netherlands), polyethylene tubes by Greiner (Alphen a/d Rijn, The Netherlands), and glass fiber filters (GF/B) by Whatman (Maidstone, UK). Rialuma scintillation cocktail was obtained from Lumac (Olen, Belgium).

Preparation of membrane-bound receptors

Calf brains (minus the cerebellum), obtained from the slaughterhouse, were stored at −80°C. The material was thawed and homogenized in 5 volumes (v/w) of ice-cold 0.32 *M* sucrose using a glass-PTFE Potter-Elvehjem homogenizer (R.W.18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was carefully decanted and centrifuged for 60 min at 100,000 *g* at 4°C. The pellet was washed twice with 50 m*M* sodium phosphate buffer (pH 7.4) containing 1 m*M* EDTA followed by centrifugation for 30 min at 100,000 *g* at 4°C. The final pellet was suspended in 5 volumes of the same buffer, rapidly frozen in liquid nitrogen and lyophilized for 48 h. Lyophilized material could be stored at

−20°C for at least 1 year with retention of receptor activity, which is in agreement with the results of Lund (1981).

Solubilization of brain membranes

The lyophilized brain membranes were suspended to a protein concentration of 8mg/ml in ice-cold 50 m*M* Tris-HCl (pH range 6.7-8.2, 22°C) (buffer T) supplemented with protease inhibitors (1 m*M* benzamidine HCl and 200 µg/ml bacitracin), and 1 m*M* EDTA. The proteins were solubilized on ice by dropwise addition of 20 mg/ml deoxycholate (pH 7.6), to a final detergent concentration of 2 mg/ml. After 30 min, non-solubilized material was removed by centrifugation at 100 000 *g* for 1 h at 4°C. To study the effect of KCl on the solubilization efficiency, 0.0-1.0 *M* KCl was added to the 50 m*M* Tris-HCl buffer (pH 7.6, 22°C) supplemented with protease inhibitors and EDTA as described above.

The supernatant containing solubilized receptors was immediately used in the binding assays and had a final protein concentration of approximately 5 mg/ml when the proteins were solubilized at pH 7.6 with 0.5 *M* KCl added.

Binding assay

For the determination of the affinity of membrane-bound receptors for flunitrazepam (FNM) and the number of binding sites, samples of the receptor suspension containing 80 µg protein were incubated in polyethylene tubes for 45 min at 0-2°C with 20 µl aliquots of [^{3}H]FNM stock solutions (in buffer T), giving final concentrations ranging from 0.1 to 10 n*M*. The total volume of the incubation mixture was adjusted to 200 µl with buffer T. To determine nonspecific binding, the incubation was also done in the presence of an excess of unlabeled ligand, 5 µ*M* lorazepam. After the incubation, of 15 µl of a solution of 33 mg/ml bovine globulins and 85µl of a 360 mg/ml PEG-6000

solution were added to give a total of 100 µl PEG/globulins in buffer T. The incubation was continued for another 12 min and then stopped by the addition of 3 ml ice-cold buffer T. The mixture and two 3 ml rinsing portions of ice-cold buffer T were filtered over Whatman GF/B filters, after which the filters were transferred into scintillation counting vials, shaken in a 3.5 ml scintillation cocktail for 2 h, and subjected to 5 min liquid scintillation counting (Packard Tri-Carb 4000, Downers Grove, IL, USA). The binding assay for solubilized receptors was done essentially as above, except that [^{3}H]FNM concentrations up to 20 n*M* were used.

The equilibrium dissociation constant (K_d) for the labeled ligand and the maximal number of operative binding sites per mg protein (B_{max}) were calculated using the EBDA-LIGAND computer fitting program (Version 4.0; Biosoft, Cambridge, UK), based upon the LIGAND program originally developed by Munson and Rodbard (1980), considering a one-site binding model.

Protein determination

The amount of protein in the receptor preparations was determined by a modified Lowry method (Clark, 1984) after protein precipitation by the addition of trichloroacetic acid to avoid interference of detergent and Tris (Bensadoun and Weinstein, 1976). Bovine serum albumin was used as standard and was treated as other samples.

2.3 Results and Discussion

Deoxycholate is a suitable detergent for efficiently solubilizing GABA- and benzodiazepine receptors from calf brain (Asano and Ogasawara, 1980 and Asano and Ogasawara, 1981).

However, since deoxycholate concentrations higher than about 10 mg/ml inhibit benzodiazepine receptor binding activity (Figure 2.1), solubilization was done at the lowest concentration (2 mg/ml) still capable of solubilizing most of the brain protein, to minimize the risk of irreversibly damaging the receptor protein.

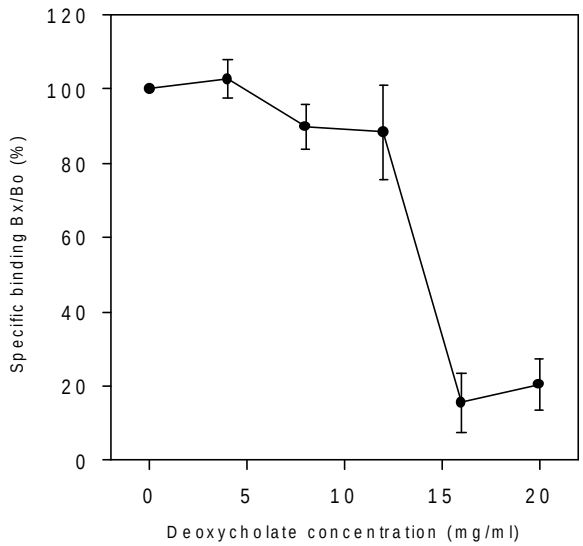

Figure 2.1 Influence of the concentration of sodium deoxycholate on the percentage specific binding (Bx/Bo; where Bx is the specific binding (total binding − nonspecific binding) at a certain deoxycholate concentration and Bo is the specific binding when no detergent is present) of the solubilized $GABA_A$-benzodiazepine receptor. The [^{3}H]FNM concentration added to the receptor suspension was 1 n*M*. Bars represent the standard deviation of measurement (n=5). During the incubation the pH of the mixture was 7.6 while no KCl was present.

The effect of the pH on the solubilization of the benzodiazepine receptor is shown in Figure 2.2. The decrease in specific binding of [^{3}H]flunitrazepam ([^{3}H]FNM) to the benzodiazepine at higher pH values is probably not caused by a lower solubilization efficiency of these binding sites, since the amount of solubilized protein is in all cases around 60%, but may be due to (partly) inactivation of the receptor binding sites. This pH effect was also described by Ensing and de Zeeuw (1984) for the muscarinic acetylcholine receptor, where it was shown that the equilibrium association constants (K_a) for pirenzepine were decreasing at increasing pH, while for dexetimide the K_a values were decreased when the pH was lower than pH 6.6 or higher than 7.8.

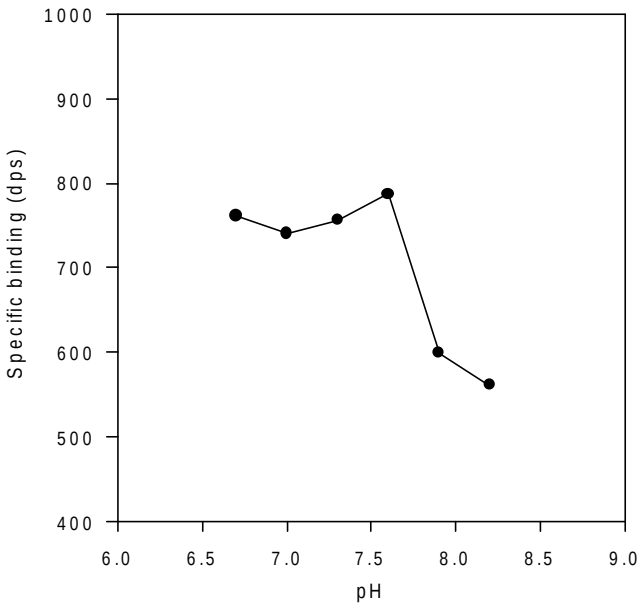

Figure 2.2 Effect of the pH on the solubilization of the benzodiazepine receptor, measured as the amount of specific binding of 10 n*M* [^{3}H]FNM. The values are the averages of two experiments.

The presence of KCl increased the amount of specific binding of [^{3}H]FNM and was reaching a plateau at 0.5 *M* (Figure 2.3). Yet, it did not increase the amount of solubilized protein (~60%). It has been suggested that solubilization in the presence of a high salt concentration (especially KCl) solubilizes a different class of benzodiazepine receptors which are insensitive to solubilization without salt (Lo *et al.*, 1982).

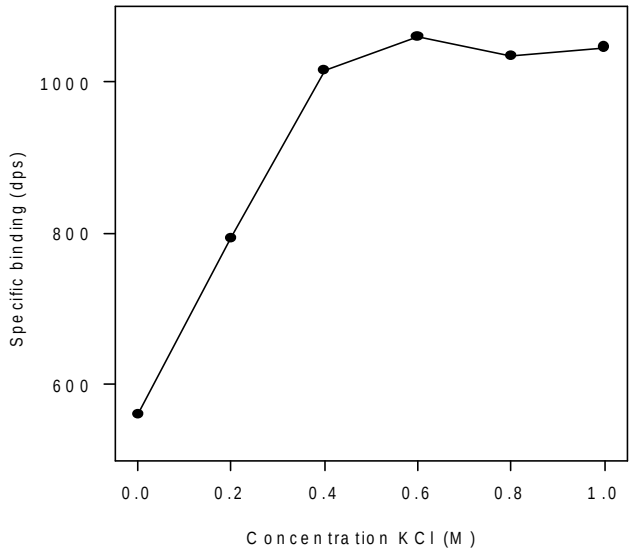

Figure 2.3 Effect of KCl on the solubilization of the benzodiazepine receptor measured as the amount of specific binding of 10 n*M* [^{3}H]FNM. The values are the averages of two experiments.

Optimal conditions for solubilization of the benzodiazepine receptor were therefore determined to be 2 mg/ml deoxycholate in 50 m*M* Tris-HCl buffer (pH 7.6) supplemented with protease inhibitors (1 m*M* benzamidine HCl and 200 µg/ml bacitracin) and 0.5 *M* KCl. This resulted in a protein recovery of 59±9% (*n*=6), while 54±6% [^{3}H]FNM binding sites were recovered in the

soluble fraction. Though it has been reported that more protein could be solubilized using 5 mg/ml deoxycholate in combination with 150 m*M* KCl protease inhibitors, the amount of active solubilized [^{3}H]FNM binding sites did not increase (Janssen.*et al.*, 1996).

The solubilized receptor bound [^{3}H]FNM with a K_d of 2.7±1.2 n*M* and a B_{max} of 0.40±0.04 pmol/mg protein (n=10), compared to a K_d of 1.46±0.17 n*M* and a B_{max} of 0.85±0.11 pmol/mg protein for membrane-bound receptors. Figure 2.4 shows representative saturation binding curves for the membrane-bound and solubilized benzodiazepine receptors.

Solubilization of the $GABA_A$-benzodiazepine receptor with 2 mg/ml sodium deoxycholate in 50 m*M* Tris-HCl buffer (pH 7.6) supplemented with the protease inhibitors, 1 m*M* EDTA and 0.5 *M* KCl did not greatly affect receptor stability over a period of one day. However, the binding activity of the solubilized benzodiazepine receptor was lost within a week when stored at temperatures of –20°C or higher (results not shown), but was retained for at least three months upon storage at –80°C (Janssen *et al.*, 1997).

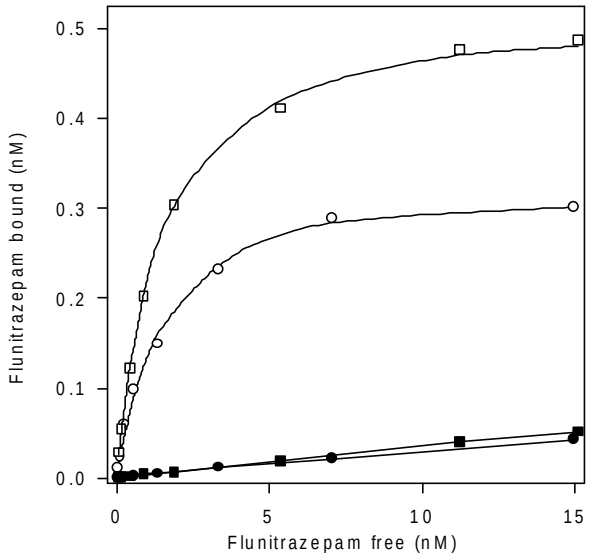

Figure 2.4 Saturation curves of membrane-bound receptors ( ) and solubilized receptors (O). Open symbols represent specific binding and closed symbols the nonspecific binding.

2.4 Discussion

Solubilization of benzodiazepine receptors can result in a high-affinity soluble receptor preparation as described in this Chapter, although more than 50% of the receptor binding sites are lost or inactivated, which is in agreement with the findings described by others (Hammond and Martin (1986), Asono and Ogasawara (1980) and (1981), and Stephenson and Olsen (1982). Nevertheless, solubilization is a necessary first step in the reconstitution of membrane proteins (see Chapter 3). We selected deoxycholate at a low concentration (2 mg/ml) and addition of 0.5 *M* KCl, because these conditions preserved the highest amount of active benzodiazepine receptor sites.

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Chapter 3

Reconstitution of the Solubilized Bovine Brain Benzodiazepine Receptor

3.1 Introduction

Since the $GABA_A$-benzodiazepine receptor (like many other membrane proteins) is unstable in detergent solutions (see also Chapter 2), the (active) receptor is preferably studied after reconstitution in lipid vesicles. Various reports have appeared in recent years on the functional reconstitution of receptor proteins, under which the benzodiazepine receptor (Schoch *et al.*, 1984, Sigel *et al.*, 1985, Hirouchi *et al.*, 1987 and Dunn *et al.*, 1989), the δ opiate receptor (Fujioka *et al.*, 1988, Scheideler and Zukin, 1990 and Gomathi and Sharma, 1993), the μ opiate receptor (Gioannini *et al.*, 1993), the myometrial oxytocin receptor (Klein and Fahrenholz, 1993) and the acetylcholine receptor (Lindstrom *et al.*, 1980 and Anholt *et al.*, 1982). Dunn *et al.* (1989) have reconstituted the β-octyl-glucoside-solubilized $GABA_A$-benzodiazepine receptor with size-exclusion chromatography (gel filtration) as a tool for detergent depletion. Other methods included amongst others dialysis of a mixture of Triton X-100- or CHAPS-solubilized benzodiazepine receptors and lipids (Sigel *et al.*, 1985) and adsorption of Triton X-100 (from a mixture of Triton X-100-solubilized benzodiazepine receptors and soybean lipids) to

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Modified version of:
Viel, G.T., Yang, Q., Lundahl, P., Ensing, K. and de Zeeuw, R.A. (1997) Size-exclusion chromatographic reconstitution of the bovine brain benzodiazepine receptor. Effects of lipid environment on the binding characteristics. *J. Chromatogr. A* **776**, 101-107.

Bio-Beads SM-2 (Schoch *et al.*, 1984). In most cases, however, the reconstituted receptors bound their radiolabeled ligands with lower affinities (factor 2-6). The use of specific phospholipids during the reconstitution procedure might improve the recovery of pharmacologically active receptors, as described by Balen *et al.* (1994) for the D-1 dopamine receptor, where phospholipid vesicles made of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in a ratio PE:PC of 1:2 gave the highest recovery of active receptor binding sites.

This chapter describes the reconstitution of sodium deoxycholate-solubilized benzodiazepine receptors into vesicles of various lipid compositions. These proteoliposomes were tested for flunitrazepam (FNM) affinity and binding capacity, the effect of freezing and thawing on the FNM binding parameters, and the long-term stability of the reconstituted receptor. The purpose was to assess the efficiency of the chromatographic reconstitution procedure with a variety of lipid mixtures. The proteoliposomes will be used as a tool in the development of a non-radioactive receptor assay for benzodiazepines (see Chapter 4-6).

3.2 Materials and Methods

Materials

Bovine serum albumin (type 5, 96-99%), bovine globulins (Cohn fractions II and III), benzamidine hydrochloride (97%), bacitracin (50,000 units/g), bovine brain lipids (type I (BPL), 50-60% phosphatidylserine), cholesteryl hemisuccinate (free acid) and sodium deoxycholate (>95%) were obtained from Sigma (St. Louis, MO, USA). Cholic acid (98% or >99%) was purchased from Sigma or Fluka (Buchs, Switzerland), egg L-α-lecithin-phosphatidylcholine (>95% PC) and soybean lipids (asolectin, i.e. crude soybean extract containing

approx. 20% PC) were from Avanti Polar Lipids (Alabaster, AL, USA). [*N-methyl*-^{3}H]flunitrazepam (82.0 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Lorazepam was kindly provided by Wyeth Laboratoria (Hoofddorp, The Netherlands). Polyethylene glycol (PEG) 6000 was supplied by Genfarma (Maarssen, The Netherlands), Sephadex™ G-50 Medium by Pharmacia Biotech (Uppsala, Sweden), polyethylene tubes by Greiner (Alphen a/d Rijn, The Netherlands), and glass fiber filters (GF/B) by Whatman (Maidstone, UK). Rialuma scintillation cocktail was obtained from Lumac (Olen, Belgium). Other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Preparation of membrane-bound receptors

Calf brains (minus the cerebellum), obtained from the slaughterhouse, were stored at −80°C. The material was thawed and homogenized in 5 volumes (v/w) of ice-cold 0.32 *M* sucrose, using a glass-PTFE Potter-Elvehjem homogenizer (R.W.18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands) at 1000 *g* for 10 min at 4°C. The supernatant was carefully decanted and centrifuged for 60 min at 100,000 *g* at 4°C. The pellet was washed twice with 50 m*M* sodium phosphate buffer (pH 7.4) containing 1 m*M* EDTA followed by centrifugation for 30 min at 100,000 *g* at 4°C. The final pellet was suspended in 5 volumes of the same buffer, rapidly frozen in liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkerød, Denmark) for 48 h. Lyophilized material could be stored at −20°C for at least 1 year with retention of receptor activity, which is in agreement with the results of Lund (1981).

Solubilization of brain membranes

The lyophilized brain membranes were suspended to a protein concentration of 8mg/ml in ice-cold 50 m*M* Tris-HCl (pH 7.4, 22°C) (buffer T) supplemented with protease inhibitors (1 m*M* benzamidine HCl and 200 µg/ml bacitracin), 0.5 *M* KCl and 1 m*M* EDTA. The proteins were solubilized on ice by dropwise addition of 20 mg/ml deoxycholate (pH 7.6), to a final detergent concentration of 2 mg/ml. After 30 min, non-solubilized material was removed by centrifugation at 100 000 *g* for 1 h at 4°C.

The supernatant containing solubilized receptors was directly used in the reconstitution procedures and had a protein concentration of approximately 5 mg/ml.

Reconstitution of the solubilized receptor by size-exclusion chromatographic detergent depletion

The procedure followed for the reconstitution of membrane proteins was essentially as described for liposome preparation by Brunner *et al.* (1976), for proteoliposome preparation by Lundahl and Yang (1991) and as applied to the human red cell glucose transporter by Brekkan *et al.* (1996a). Briefly, lipid mixtures of the compositions specified in the Results section were dissolved in chloroform and subjected to rotary evaporation in a round-bottomed flask. Redissolution and evaporation was repeated twice with diethylether and the lipid film was flushed with nitrogen. The lipids were dispersed with 125 m*M* sodium cholate in 50 m*M* Tris-HCl (pH 8.0, 22°C) to obtain a lipid concentration of 75 mg/ml (approximately 100 m*M*). A 1.5 ml aliquot of this solution was mixed with 2.5 ml of deoxycholate-solubilized membranes and applied with a flow of 1.5 ml/min to a 39 cm × 2 cm Sephadex™ G-50 M gel bed in buffer T supplemented with 0.1 *M* KCl at 4°C. The void volume

fractions containing the proteoliposomes were collected and pooled, and the amounts of protein and phosphorus were determined as described below.

To study the effect of freezing and thawing of the proteoliposomes on the receptor, the tube containing the proteoliposome suspension obtained after the Sephadex chromatography was immersed for 5 min in a CO_2(s)-ethanol bath (approx. −70°C) and then for 5 min in a water bath at 25°C. The suspension was vigorously mixed by vortex and the freeze-thawing cycle was repeated once. Proteoliposomes and freeze/thawed proteoliposomes were used immediately in the binding experiments. Alternatively, (freeze/thawed) proteoliposomes can be stored at −20°C for at least 2 months without loss of receptor binding (see Results section). The size distribution of the formed proteoliposomes was determined with dynamic laser light scattering using a particle sizing system (Model 370, Nicomp, Santa Barbara, CA, USA)

Binding assay

For the determination of the affinity of reconstituted receptors for flunitrazepam (FNM) and the number of binding sites, samples of the receptor suspension containing 80 µg protein were incubated in polyethylene tubes for 45 min at 0-2°C with 20 µl aliquots of [^{3}H]FNM stock solutions (in buffer T), giving final concentrations ranging from 0.1 to 10 n*M*. The total volume of the incubation mixture was adjusted to 200 µl with buffer T. To determine nonspecific binding of [^{3}H]FNM, the receptor was also incubated with an excess of unlabeled ligand (5 µ*M* lorazepam). After the incubation, 15 µl of a solution of 33 mg/ml bovine globulins and 85µl of a 360 mg/ml PEG-6000 solution were added (to give a total of 100 µl PEG/globulins in buffer T). The incubation was continued for another 12 min and then stopped by the addition of 3 ml ice-cold buffer T. The mixture and two 3 ml rinsing portions of ice-cold buffer T were subsequently filtered over Whatman GF/B filters, after which the filters were transferred into scintillation counting vials, shaken in 3.5 ml

scintillation cocktail for 2 h, and subjected to 5 min liquid scintillation counting (Packard Tri-Carb 4000, Downers Grove, IL, USA). The binding assay for solubilized receptors was done essentially as above, except that [^{3}H]FNM concentrations up to 20 n*M* were used. The equilibrium dissociation constant (K_d) for the labeled ligand and the maximal number of functional binding sites per mg protein (B_{max}) were calculated using the EBDA-LIGAND computer fitting program (Version 4.0; Biosoft, Cambridge, UK), based upon the LIGAND program originally developed by Munson and Rodbard (1980), considering a one-site binding model.

Protein and phosphorus determinations

The amount of protein in the receptor preparations was determined by a modified Lowry method (Clark, 1984) after protein precipitation by the addition of trichloroacetic acid to eliminate interference of detergent and Tris (Bensadoun and Weinstein, 1976). Bovine serum albumin was used as standard and was treated as the other samples. Phospholipids were determined as phosphorus as described by Bartlett (1959).

3.3 Results and Discussion

Reconstitution of solubilized benzodiazepine receptors and FNM affinity to the benzodiazepine receptor

The deoxycholate-solubilized benzodiazepine receptor (approx. 5 mg protein/ml and 20-40 m*M* phospholipids) was reconstituted by size-exclusion chromatography on Sephadex™ G-50 M, in the presence of endogenous bovine brain lipids and additions of soybean lipids (Aso), brain phospholipids (BPL), cholesteryl hemisuccinate (Cholh) and egg phosphatidylcholine (EPC) in various combinations (Table 3.1). Only slight differences in reconstitution efficiency were seen when other lipid combinations were used. The

concentration of KCl in the eluent affected the reconstitution efficiency: with 0.1 *M* KCl, 52±7% of the total solubilized proteins were incorporated into proteoliposomes composed of 100 m*M* EPC (and endogenous brain lipids), while only 30-40% of the proteins were incorporated in the presence of 0.5 *M* KCl. The phospholipid concentration was between 11 and 17 m*M* in all preparations tested.

Table 3.1
Reconstitution of the benzodiazepine receptor using 0.1 *M* KCl in the eluent and the effect of freezing and thawing on the binding properties of the reconstituted receptor

Lipids added[a]	Specific [^{3}H]FNM binding[b]		Specific [^{3}H]FNM binding[b] after freeze-thawing	
	B_{max} (pmol/mg protein)	K_d (n*M*)	B_{max} (pmol/mg protein)	K_d (n*M*)
Aso	0.25	2.9	0.42	2.8
Aso/BPL/Cholh (7:3:1)	0.16	2.1	0.26	2.6
EPC/Aso/BPL (3.5:3.5:3)	0.12	2.4	0.20	2.7
EPC (100 m*M*)	0.09	4.0	N.D.[c]	N.D.[c]
EPC/BPL/Cholh (7:3:1)	0.07	2.0	0.20	3.8
Aso/BPL (7:3)	0.04	2.2	0.25	3.2
EPC/Aso/BPL/Cholh (3.5:3.5:3:1)	0.04	1.4	N.D.[c]	N.D.[c]
Membrane homogenate[d]	0.85±0.11	1.5±0.2	N.D.[c]	N.D.[c]
Solubilized receptors[d]	0.40±0.04	2.7±1.2	N.D.[c]	N.D.[c]

[a] Lipids added in the reconstitution experiments. The ratio (w/w) of the lipids is shown in parentheses. The total lipid concentration was approximately 100 m*M*. Aso = asolectin (soybean lipids); BPL = brain phospholipids; EPC = egg phosphatidylcholine; Cholh = cholesteryl hemisuccinate.

[b] The values are the averages of 2-4 reconstitution experiments. The average relative SEM was ±40% for the B_{max} values and ±13% for the K_d values.

[c] N.D.; not determined.

[d] Values for comparative purposes

Saturation binding experiments were done to examine the [^{3}H]FNM binding characteristics of the proteoliposomes. Although the lipid composition of the liposomes was changed and various lipid sources were used, the K_d values were

not dramatically affected, but were similar to those obtained with the solubilized receptor (Table 3.1). Larger variations were found in the B_{max} values, which fell between 0.04 and 0.25 pmol/mg protein (10-63% of the B_{max} value found with the solubilized receptor).

For a given preparation of solubilized material the differences in B_{max} after reconstitution were small, in contrast to the variation seen when the receptors originated from different solubilizates (results not shown).

When the benzodiazepine receptor was reconstituted with the co-solubilized endogenous brain membrane lipids only, the binding was almost completely lost, although still 60% of the total protein was incorporated in the formed liposomes. Considering the good K_d value and the higher amount of binding sites (B_{max}) found when the benzodiazepine receptor was reconstituted in asolectin proteoliposomes, we selected asolectin as the lipid source in further reconstitution experiments.

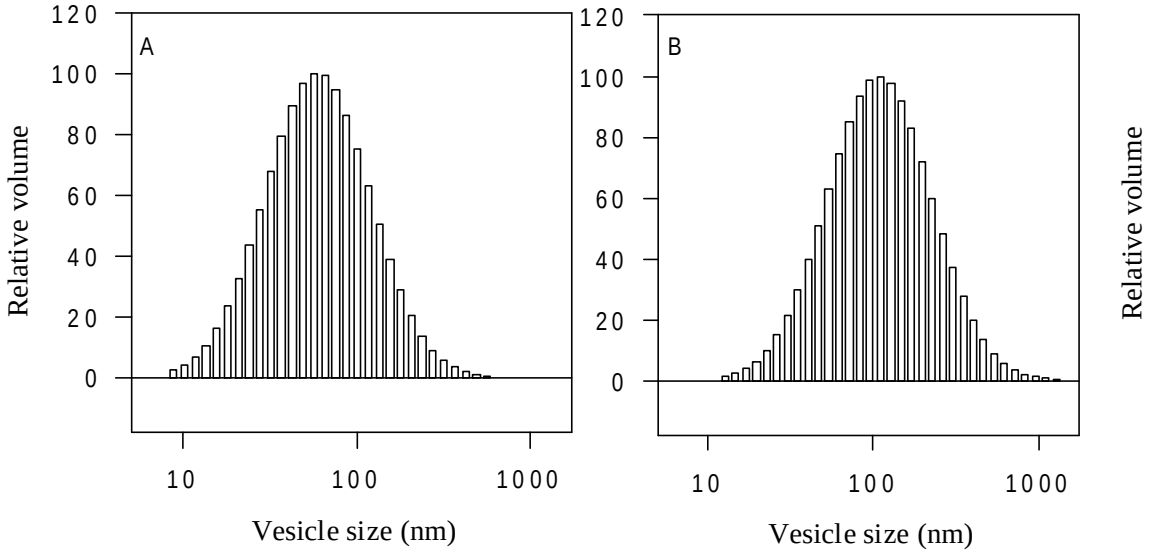
Effect of freeze/thawing of proteoliposomes on the [^{3}H]FNM binding to reconstituted benzodiazepine receptor

During freeze/thawing, (proteo)liposomes fuse to form larger (proteo)liposomes, as indicated by increasing inner volumes (Pick, 1981; MacDonald and MacDonald, 1993 and Brekkan *et al.*, 1996b). This may provide a more favorable environment for a large multireceptor complex such as the $GABA_A$-benzodiazepine receptor. The proteoliposomes were therefore freeze-thawed, which resulted in increasing B_{max} values, whereas the changes K_d values were relatively small (Table 3.1).

The highest B_{max} value obtained was 0.42±0.17 pmol/mg protein for proteoliposomes prepared with soybean lipids (asolectin), which is similar to the B_{max} found with solubilized receptor.

The B_{max} values doubled on the average although the variations between repetitive experiments were large.

The size distribution of the proteoliposomes was determined by dynamic laser light scattering. Figure 3.1A shows the size distribution of asolectin made proteoliposomes. The mean diameter of the vesicles was determined to be 75 nm. 99% of all vesicles were smaller than 290 nm. Proteoliposomes made from other lipid combinations showed roughly the same size distribution. A more homogeneously sized liposome population might be formed when the formed



proteoliposomes would be extruded through membrane filters (Turánek, 1994).

Figure 3.1 Analysis of the vesicle size of asolectin-made proteoliposomes as determined by dynamic laser light scattering. A: vesicle size distribution of freshly prepared proteoliposomes and B: vesicle size distribution of the proteoliposomes after a freezing and thawing cycle. The proteoliposomes contained approx. 100 m*M* asolectin.

The size distribution of the asolectin proteoliposomes after freezing and thawing is shown in Figure 3.1B, where the mean size of proteoliposomes made with asolectin was determined to be 150 nm, which is twice the size of

the originally prepared proteoliposomes. 99% of the proteoliposomes were smaller than 550 nm. For proteoliposomes made with other lipid combinations, the increase of size was smaller (1.2-1.5 times increase). The larger size of the vesicles after freezing and thawing might be the cause of the increase in B_{max} since a high curvature of the membranes of the original vesicles may give (partial) inactivation of the receptor protein. However, the higher B_{max} values seen with proteoliposomes made with lipids other than asolectin cannot just be explained by the (smaller) increase in size of the vesicles. Therefore, other (unknown) factors than particle size seem to plat a role in this phenomenon.

Stability of the reconstituted benzodiazepine receptor

Whereas lyophilized membrane-bound benzodiazepine receptors can be stored for at least one year at −20°C with retention of the binding characteristics, solubilized receptors are more susceptible to inactivation of the receptor binding due to the presence of detergents, although Janssen (1997) described that solubilized receptors retained their high binding capacity for at least 2 months when stored at −80°C.

It was anticipated that the stability of the receptor would be increased after reconstitution. We examined the stability of the reconstituted receptor over a 2 month period under a variety of storage conditions. At 4°C, about 20% of the binding capacity of the reconstituted benzodiazepine receptor was retained after 2 weeks (Figure 3.2).

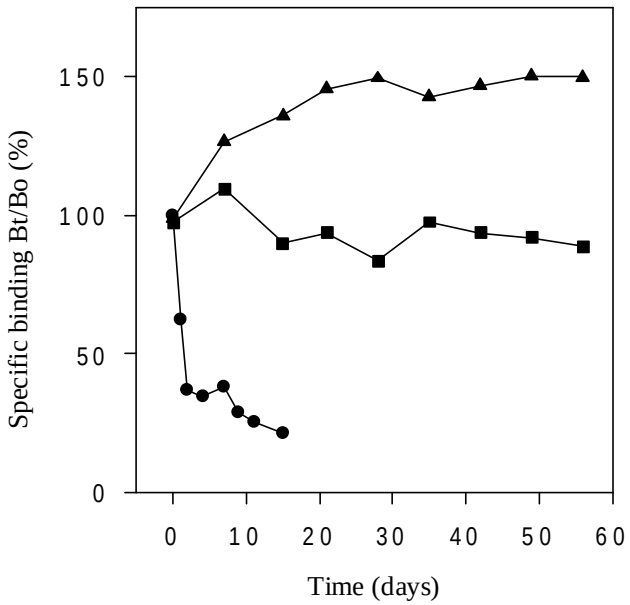


Figure 3.2 Long-term stability of reconstituted $GABA_A$-benzodiazepine receptors stored at 4°C (•), −20°C (◊) or −80°C (▲). B_t is the specific binding at day t, B_0 is the specific binding at day 0 (freshly prepared proteoliposomes). The [^{3}H]FNM concentration added to the proteoliposome suspension containing the benzodiazepine receptor was 1 n*M*. The receptors were reconstituted using egg phosphatidylcholine-asolectin-brain phospholipids (3.5:3.5:3), and the proteoliposomes were formed during gel filtration on Sephadex G-50 M in buffer T supplemented with 0.1 *M* KCl.

At −20°C, −80°C, the binding capacity was essentially retained during a period of 2 months. The increase in the B_{max} value observed when the proteoliposomes were stored at −80°C was probably the result of proteoliposome fusion upon freezing and thawing, as described above. Alternatively, proteoliposomes could be lyophilized and stored at −20°C for a 2 month period without loss of the binding parameters (result not shown).

3.4 Conclusion

Reconstitution of membrane proteins into liposomes offers the possibility to study these proteins in a stable environment, similar to that in the native membrane. Although the amount and type of lipids present in the bilayer are important to the activity of the (receptor) protein, it is difficult to predict the optimal lipid composition in the proteoliposomes. Hammond and Martin (1987) showed that the addition of phosphatidylserine at concentrations as low as 5 μM during and after the solubilization of the benzodiazepine receptor from synaptic membranes, enhanced flunitrazepam binding. According to Moynagh and Williams (1992), both phosphatidylserine and phosphatidylinositol were effective in preserving and even stimulating the (peripheral) benzodiazepine receptor activity. The use of mild solubilization conditions may preserve various endogenous and probably critical phospholipids. The other lipids added during the reconstitution experiments showed no dramatic differences in the binding characteristics of the benzodiazepine receptor, although the best results were obtained with asolectin, a crude lipid containing soybean extract. In contrast to Hammond and Martin (1987) and Moynagh and Williams (1992), addition of extra phosphatidylserine (a major constituent in type I brain phospholipids) did not increase the number of operative benzodiazepine binding sites.

Since the $GABA_A$-benzodiazepine receptor complex is large (about 300 kDa), receptor activity may be reduced in smaller-size vesicles. The results after freezing and thawing which gave larger-size vesicles resulted in more binding sites, although particle size may not be the only factor involved in this phenomenon. Alternatively, freezing and thawing could reorganize the membrane and facilitate better incorporation of the receptor complex in the bilayer.

In this study, our goal was to obtain the active benzodiazepine receptor reconstituted in lipid vesicles. Although the amount of binding sites (B_{max}) of the benzodiazepine receptor found after reconstitution in asolectin liposomes is considerably lower than that found in crude membrane homogenate, which is caused by losses of protein and/or receptor activity during solubilization and, to a less extent reconstitution (also found by other investigators), the K_d values obtained were lower (thus a higher affinity) than described by others (Dunn *et al.* 1989, Sigel *et al.* 1985, and Schoch *et al.* 1984). Therefore, we were able to successfully reconstitute the benzodiazepine receptor in a stable form, with preservation of high affinity binding sites. The proteoliposomes could be an essential element, as a carrier for the benzodiazepine receptor and nonradioactively labeled entities, in the development of a heterogeneous nonradioactive receptor assay (see Chapters 4-6).

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Chapter 4

Preparation and Characterization of Fluorescent Labeled (Proteo)liposomes

4.1 Introduction

Receptor assays traditionally make use of radioligands for the detection of the binding of these ligands or analytes. Recently, other types of labels for receptor assays have been developed such as fluorescent labels (reviewed by Baindur and Triggle, 1994a and 1994b), biotin labeled ligands (Chen *et al.*, 1992, and Takeuchi *et al.*,1992) and enzymes (Santos and Rechnitz, 1990 and Hallowell and Rechnitz, 1987). The main problem of using these labels lies in the fact that in almost all cases the binding characteristics of the labeled ligand were significantly reduced in comparison with the parent compound (precursor).
Janssen (1997) described the affinities of fluorescent-labeled 1,4-benzodiazepines, fluorescent-labeled 1,2-annelated 1,4-benzodiazepines and their precursors, for the benzodiazepine receptor (Table 4.1).

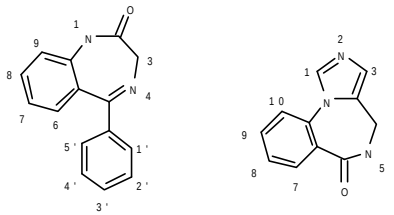


Figure 4.1 Basic structures of 1,4 benzodiazepines and 1,2 annelated benzodiazepines.

For benzodiazepines, only labeling at position 1 did not much influence the binding activity (see Figure 4.1 for the structures of 1,4-benzodiazepines and

1,2-annelated 1,4-benzodiazepines). High affinities could be obtained by using a small fluorophore and a spacer to optimize the distance between the fluorophore and the drug. A further requirement for a suitable fluorescent ligand is that if hydrolysis occurs, the resulting products should have no affinity for the receptor binding sites.

To circumvent these difficulties, we tried to construct a different fluorescent receptor assay, where the ligand is immobilized to a solid support (see Chapters 5 and 6 for details) and the receptor is (indirectly) labeled by reconstitution in a liposome containing fluorescent lipids. Since the constituents of a membrane (and of proteoliposomes) may have some autofluorescence in the ultraviolet and lower visible range (250-400 nm), the fluorescent lipid probes should have an excitation (and emission) wavelength that should be as high as possible, preferably exceeding 450 nm. Of these, fluorescein (λ_{ex}=492), rhodamine B (λ_{ex}=550 nm) and Bodipy FL (λ_{ex}=505 nm) fulfill this requirement (see Figure 4.2 for the structures of some commonly used fluorophores).

A second requirement is that the fluorophore must have high fluorescence intensity in an aqueous environment, since the receptor assay is (normally) done in such a medium. However, when the receptor-bound fraction is measured, organic solvents may also be used, with or without prior dissociation of the bound fraction (Janssen, 1997). The use of organic media also might improve the fluorescence characteristics of the probes.

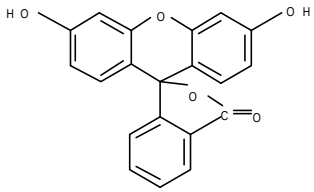
Furthermore, the detection limit of the fluorescent lipid probe is dependent on the instrumentation used. Chromatographic fluorescence detectors are known to usually be more sensitive than static detectors (Janssen, 1997).

In this chapter we investigate which (commercially available) fluorescent lipids may be suitable for use as a label in fluorescent liposomes. We therefore selected three fluorescent labels (Figure 4.3 and Table 4.2): N-(5-dimethylamino-naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (dansyl DHPE), N-(5-fluoresceinthiocarbamoyl)-1,2-dihexa-decanoyl-sn-glycero-

3-phosphoethanolamine, triethyl-ammonium salt (fluorescein DHPE) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexa-decanoyl-sn-glycero-3-phosphocholine (β-Bodipy Fl C_5HPC).

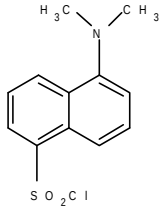
Table 4.1 K_I-values of fluorescent-labeled 1,4 benzodiazepines, fluorescent-labeled 1,2-annelated 1,4 benzodiazepines and their precursors (from Janssen, 1997).

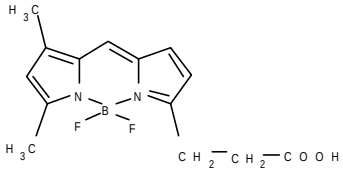
Precursors and fluorescent derivatives	K_i
Oxazepam	16.7 n*M*
Mmc-O-CO-$(CH_2)_2$-CO-oxazepam	>1 m*M*
Lormetazepam	1.2 n*M*
Mmc-O-CO-$(CH_2)_2$-CO-lormetazepam	114 n*M*
Nitrazepam	7.4 n*M*
7-aminonitrazepam (Ro5-3072)	470 n*M*
Dansyl-Ro5-3072	>1 m*M*
Flurazepam	10.4 n*M*
Didesethylflurazepam (Ro7-1986)	4.9 n*M*
Dansyl-Ro7-1986	167 n*M*
Bodipy-Ro7-1986	67 n*M*
NBD-$(CH_2)_2$-CO-Ro7-1986 (Velazquez *et al.*, 1989)	51 n*M*
NBD-$(CH_2)_5$-CO-Ro7-1986 (Velazquez *et al.*, 1989)	132 n*M*
Fluorescein-Ro7-1986 (McCabe *et al.*, 1990)	163 n*M*
AMCA-Ro7-1986 (Takeuchi and Rechnitz, 1991)	8.6 n*M*
Flumazenil	0.6 n*M*
Desethylflumazenil (Ro15-3890)	>1 m*M*
Mmc-Ro15-3890	121 n*M*
Mmc-O-CO-$(CH_2)_3$-Ro15-3890	6.5 n*M*
Fluorescein-NH-$(CH_2)_3$-Ro15-3890 (McCabe *et al.*, 1990)	63 n*M*
NBD-NH-$(CH_2)_3$-Ro15-3890 (Havunjian *et al.*, 1990)	5.7 n*M*
1021-S (Taguchi and Kuriyama, 1984)	0.4 n*M*
NBD-1021-S (Velazquez *et al.*, 1989)	85 n*M*



Fluorescein: λ_{ex} = 492 nm, λ_{em} = 520 nm

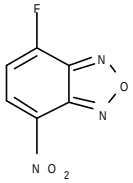
Rhodamine B: λ_{ex} = 550 nm, λ_{em} = 585 nm

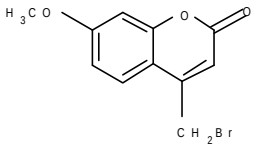




Dansylchloride: λ_{ex} = 340 nm, λ_{em} = 480-520 nm

Bodipy Fl-3-propionic acid: λ_{ex} = 505 nm, λ_{em} = 511 nm



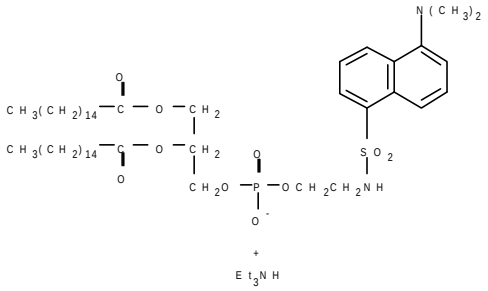


F-NBD: λ_{ex} = 470 nm, λ_{em} = 530 nm

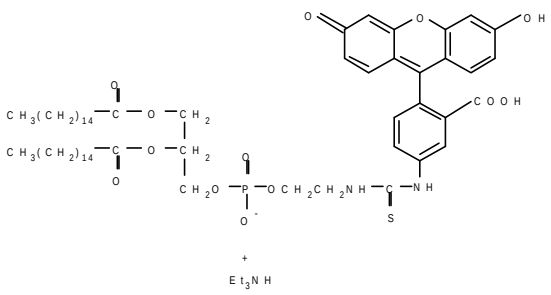
BrMmc: λ_{ex} = 315 nm, λ_{em} = 400 nm

Figure 4.2 Structures of some commonly used fluorophores.

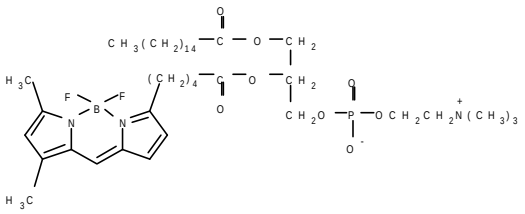
Dansyl DHPE and fluorescein DHPE are phosphatidylethanolamine with fluorescent-labeled headgroups, whereas Bodipy® FL C_5HPC is a phosphatidylcholine with a labeled acyl chain.



Dansyl DHPE



Fluorescein DHPE



Bodipy FL C_5HPC

Figure 4.3 Structures of the used fluorescent-labeled lipids.

(Proteo)liposomes containing fluorescent lipids might increase the sensitivity of the receptor assay, since more than one fluorescent lipid may participate in a single receptor-ligand binding interaction. We therefore determined the amount of labeled lipids that can be accommodated by the liposome without quenching effects. Moreover, since only a small fraction of the liposomes will bind to the immobilized ligand, the detection limit was assessed as well. Emission spectra

were recorded from the fluorescent lipids and from the fluorescent lipids incorporated in liposomes.

Table 4.2 Data table containing molecular weights (MW), absorption (ABS) and emission (EM) wavelengths and the extinction coefficient ($\varepsilon \times 10^{-3}$) for the three used fluorescent phospholipids. * : values for ε are approximate. All lipids are freely soluble in ethanol and chloroform (data from the Molecular Probes Inc. catalog, Eugene, OR, USA).

Lipid	MW	ABS (nm) (λ_{ex})	EM (nm) (λ_{em})	$\{\varepsilon \times 10^{-3}\}^*$
Dansyl DHPE	1026	336	517	4.5
Fluorescein DHPE	1183	496	519	88
Bodipy FL C_5HPC	798	503	512	80

The most suitable fluorescent-labeled phospholipid was selected by comparing the fluorescent characteristics and was used in the fluorescent receptor assay (Chapter 6).

4.2 Materials and Methods

Materials

Cholic acid (98%) was obtained from Sigma (St. Louis, MO, USA). Soybean lipids (asolectin, i.e. crude soybean extract containing approx. 20% phosphatidyl-choline) were from Avanti Polar Lipids (Alabaster, AL, USA). N-(5-dimethyl-aminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanol-amine, triethylammonium salt (dansyl DHPE), N-(5-fluorescein-thiocarbamoyl)-1,2-dihexa-decanoyl-sn-glycero-3-phosphoethanolamine, triethyl-ammonium salt (fluorescein DHPE) and 2-(4,4-

difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexa-decanoyl-sn-glycero-3-phosphocholine (β-Bodipy Fl C_5HPC) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Sephadex™ G-50 Medium was from Pharmacia Biotech (Uppsala, Sweden) and polyethylene tubes were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Preparation of (fluorescent-labeled) liposomes

The procedure followed for the preparation of (labeled) liposomes was essentially as described for liposome preparation by Brunner *et al.* (1976), for proteoliposome preparation by Lundahl and Yang (1991) and as applied to the human red cell glucose transporter by Brekkan *et al.* (1996). Briefly, 150 mg asolectin with or without fluorescent-labeled phospholipids (amount specified in the Results and Discussion section) were dissolved in approximately 20 ml chloroform and subjected to rotary evaporation in a round-bottomed flask. Redissolution and evaporation was repeated twice with diethylether and the lipid film was flushed with nitrogen. The lipids were dispersed in 2 ml 125 m*M* sodium cholate in 50 m*M* Tris-HCl (pH 8.0, 22°C) to obtain a lipid concentration of 75 mg/ml (approximately 100 m*M*). 1.5 ml of this lipid solution was applied with a flow of 1.5 ml/min to a 39 cm × 2 cm Sephadex™ G-50 M gel bed in 50 m*M* Tris-HCl (pH 7.4, 22°C) supplemented with 0.1 *M* KCl at 4°C. The void volume fractions containing the proteoliposomes were collected and pooled (approx. 15 ml total volume) and used immediately in the experiments. Phospholipids were determined as phosphorus as described by Bartlett (1959) and were 13-17 m*M*.

Solubilization and reconstitution of the benzodiazepine receptor

Solubilization of the benzodiazepine receptor and reconstitution of the receptor in asolectin proteoliposomes was done as described in Chapters 2 and 3.

Determination of the fluorescence characteristics of the fluorescent-labeled liposomes

Fluorescence emission spectra of 10-fold diluted fluorescent-labeled liposomes (dilution in 50 m*M* Tris-HCl buffer; pH 7.4) and fluorescence intensities of undiluted fluorescent-labeled liposomes were recorded on a Kontron SFM 25 spectrofluorometer (Zürich, Switzerland).

4.3 Results and Discussion

The amounts of fluorescent lipids incorporated in asolectin liposomes varied between 5 and 1000 µg per 150 mg total lipids (weight ratio range 1/30000-1/150) for fluorescein DHPE and dansyl DHPE, and between 1 and 50 µg per 150 mg total lipids (weight ratio range 1/150000-1/3000) for Bodipy FL C_5HPC. Because of the high costs of Bodipy-labeled lipids, a lower concentration of this particular lipid was used. In Figure 4.4 the fluorescence intensities of the different fluorescent liposome preparations are depicted.

Both dansyl DHPE and fluorescein DHPE showed quenching at higher concentrations of fluorescent-labeled lipids added. Due to the lower fluorescent lipid-concentration range of the Bodipy-containing liposomes, no quenching was observed in this preparation at these concentrations. The optimum excitation and emission wavelengths of the three fluorescent liposome preparations in 50 m*M* Tris-HCl buffer (pH 7.4) are reported in Table 4.3.

The fluorescence excitation- and emission spectrum of dansyl DHPE incorporated in liposomes showed a broad peak in the range of 300-650 nm, rather than a single sharp peak, which makes this probe less suitable. Since the

excitation wavelength of dansyl DHPE is 336 nm, interference of the matrix (lipids and/or proteins) can be expected at this wavelength, which ultimately could lead to increased background signals caused by autofluorescence.

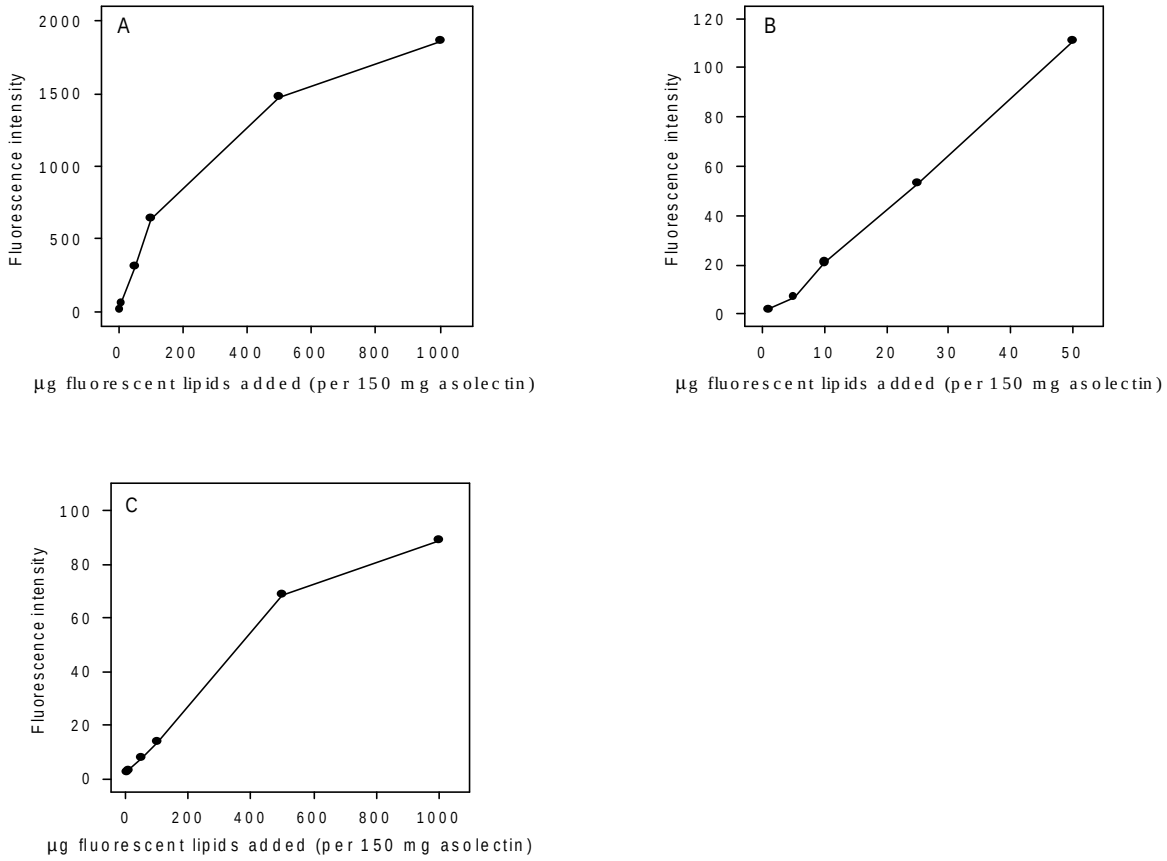


Figure 4.4 Measured fluorescence intensities (arbitrary units) of asolectin proteoliposomes containing fluorescein DHPE (A), Bodipy FL C_5HPC (B) and dansyl DHPE (C).

Table 4.3 Fluorescence characteristics of the fluorescent labeled liposomes compared to the fluorescence characteristics of the fluorescent lipids.

	λ_{ex} (nm)	λ_{em} (nm)
Dansyl DHPE	336	517
Dansyl DHPE liposomes	350	509
Fluorescein DHPE	496	519
Fluorescein DHPE liposomes	498	523
Bodipy FL C_5HPC	503	512
Bodipy FL C_5HPC liposomes	507	513

For the remaining two probes the amount of autofluorescence caused by membrane proteins and lipids was investigated. Membrane proteins containing the benzodiazepine receptor were solubilized with 0.2% deoxycholic acid (from a calf brain homogenate) (see Chapter 2 for details about the solubilization procedure), and were reconstituted in asolectin liposomes with and without fluorescent lipids (250 µg fluorescein DHPE and 50 µg Bodipy FL C_5HPC per 150 mg asolectin, respectively). In both cases the amount of autofluorescence caused by the matrix was less than 5% (results not shown).

In order to use the fluorescently labeled (proteo)liposomes in a receptor assay, it is important that low amounts of fluorescent (proteo)liposomes can be detected. We therefore determined the lowest amount of fluorescent liposomes that could still be detected. In the case of fluorescein DHPE-asolectin liposomes (250 µg fluorescein DHPE per 150 mg total lipids), the preparation could be diluted 100,000 fold, while having a fluorescence intensity significantly above background levels. With Bodipy FL C_5HPC-asolectin liposomes (50 µg Bodipy FL C_5HPC per 150 mg total lipids) a 10,000 times dilution could still be detected. It should be noted that only liposomes containing receptors will be detected. Theoretical estimations, based on a number of assumptions, indicate that (far) less than 10% of the liposomes

contain a receptor. The latter implies that the maximal dilution of proteoliposomes that can be used in the receptor assay is limited by this factor.

Therefore, fluorescein-DHPE lipids were selected for use in the fluorescent receptor assay, because a high degree of labeling of the liposomes could be achieved without quenching; in other words, small amounts of liposomes can be detected in the assay. Although the fluorescent characteristics of Bodipy-probes are superior compared to most other fluorescent labels, the costs are high. When a reasonable price per sample in the receptor assay is needed, only a small amount of the Bodipy probe can be incorporated, which will result in decreased sensitivity caused by the lowering of fluorescence intensity.

4.4 Conclusions

From the results described in this Chapter, it can be concluded that fluorescent lipids can be used to obtain highly fluorescent (proteo)liposomes. Since we expect no interactions between the receptor and the fluorescent lipids, which would allow a high degree of labeling, and because of economical reasons we selected fluorescein DHPE as the lipid probe to be used in the receptor assay. Fluorescein DHPE has a high excitation and emission wavelength, therefore it is not expected that membrane constituents disturb the detection of the fluorescent signal. Since a relatively high amount of the labeled lipids could be co-reconstituted without quenching effects, a highly diluted labeled-liposomes suspension could be easily detected. Therefore, the use of these fluorescent-labeled liposomes in a receptor assay, where a small fraction of the total proteoliposomes might bind to an immobilized ligand (see Chapters 5 and 6), should present no problems with respect to fluorescent intensity.

Further experiments should be done to optimize the procedure. The effects of pH and the use of different buffers or even organic solvents might influence the fluorescence characteristics. However, since biological molecules (e.g. receptor proteins) are abundant in the preparations used, a pH close to the physiological value (approximately pH 7.4) has to be chosen in order to assure proper functioning of these bio-molecules. A drastic pH-change could be used, however, when, for instance the receptor bound fraction is separated from the free fraction and hence good functioning of the receptor is not needed for detection of the fluorescence signal.

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Chapter 5

A Novel Benzodiazepine Radioreceptor Assay Using an Immobilized Ligand-Containing Microtiter Plate

5.1 Introduction

In traditional radioreceptor assays, all components in the assay (receptor proteins, (labeled) ligand and analyte) are incubated *in vitro* (solution-phase RA) under the proper conditions, after which the bound and free fractions of the ligand are separated (see Chapter 1). Alternatively, solid-phase receptor assays have been developed, where (in most cases) the receptors are immobilized on solid matrices, such as nitrocellulose membranes or microtiter plates.

The main advantages of using solid-phase RA over conventional solution-phase RA are:

1. Large sample numbers can be simultaneously tested with minimal effort.
2. Some investigators found that biological fluids did not interfere with the binding characteristics of the immobilized receptor, therefore, unextracted plasma or urine samples could be used (Nichols *et al.* (1992) and Suzuki *et al.* (1992).
3. The solid phase containing the immobilized receptors may be used for prolonged periods of time when used and stored under proper conditions, without significant loss of sensitivity and specificity (Suzuki *et al.*, 1992).

Suzuki *et al.* (1992) used Triton X-100 solubilized rabbit mammary prolactin (PRL) receptors, subsequently purified by concanavalin A-agarose

chromatography, and immobilized the purified receptors on a nitrocellulose membrane. Because of the low protein binding capacity of nitrocellulose membranes, purification of the receptors and the use of a labeled ligand with high specific activity (^{125}I-o-PRL) were necessary for quantitative detection of receptor binding.

Crude bovine cerebella membranes were immobilized in 96-wells microtiter plates by Nichols *et al.* (1992) for the quantitative receptor assay of endothelin. Since only 10-20 μg of membrane protein was assayed in a small volume (100 μl), considerable savings in reagents could be realized. Endothelin concentrations as low as 10^{-10} *M* could be reliably detected in serum samples and the use of a 96-wells microtiter plate facilitated the rapid determination of a large number of samples. However, since the receptor was not purified and the total amount of immobilized protein was small (resulting in a low receptor concentration), a high specific activity ligand (^{125}I-endothelin) was needed. Kaufman *et al.* (1993), immobilized the extracellular ligand binding domains of the nerve growth factor receptor (NGF receptor), basic fibroblast growth factor receptor (bFGF receptor) and the platelet-derived growth factor receptor (PDGF receptor) by biotinylation of amino groups in the extracellular domains, and coupling of the biotinylated domains to streptavidin-coated microtiter wells. All three receptors retained high affinity (0.7-1.8 n*M*) for their respective ligands. Moreover, the immobilized biotin-streptavidin-immobilized receptors could be stored at –80°C for 1 year without decreases or changes in ligand binding.

Besides immobilization of receptors, other methods for receptor assays have been developed using solid supports. For instance, Tanaka *et al.* (1992) developed a non-isotopic receptor assay for benzodiazepines using a solid-phase avidin-biotin-binding assay. Competition of enzyme-labeled (horseradish-peroxidase) avidin with immobilized biotin and biotin-labeled ligands allowed for a sensitive determination of benzodiazepines. An identical

assay was developed for the nicotinic acetylcholine receptor by the same laboratory (Chen *et al.*, 1992).

In this Chapter, we describe the development of a novel receptor assay utilizing the benzodiazepine receptor (see Figure 5.1 for the principle of the assay). A suitable benzodiazepine ligand (didesethylflurazepam) was, via a spacer arm, immobilized on a 96-wells microtiter plate (Figure 5.1A). Coupling of the ligand to the microtiter plate was done essentially as described by Sigel *et al.* (1982) and Taguchi and Kuriyama (1984) for the preparation of an affinity column for the purification of the benzodiazepine receptor. The choice of the ligand to be immobilized is very critical, since various positions on the molecule might be involved in the receptor-ligand binding, and are therefore unavailable for the immobilization reaction. Moreover, when a suitable reactive group on the molecule is available, immobilization (via a spacer arm) could reduce the affinity of the ligand for the receptor. Didesethylflurazepam has a primary amino group not involved in the receptor binding which can be used to immobilize it (via a spacer arm) to a solid phase with retention of its high affinity for the receptor, as described by Sigel *et al.* (1992).
The benzodiazepine receptor was solubilized from a calf brain homogenate (as described in Chapter 2) and reconstituted in asolectin liposomes (see Chapter 3 for details about the followed procedure), and added to the wells of the microtiter plate containing the immobilized didesethylflurazepam (Figure 5.1B). Labeled ligand ([^{3}H]flunitrazepam) added to the incubation mixture competed with the immobilized ligand for the same receptor binding site (Figure 5.1C). Increasing the amount of labeled ligand will displace more receptors initially bound to the immobilized ligand. When a fixed amount of the incubation mixture is taken from the wells and transferred to (polyethylene) tubes and an additional saturating amount of labeled ligand is added to these samples, such that the final concentration is the same in every sample, a sort of

saturation curve will be obtained, where the total amount of receptors in each sample (B_{max}) will be measured (saturation of all available binding sites by the labeled ligand), after separation of the bound and free fractions of the labeled ligand by filtration.

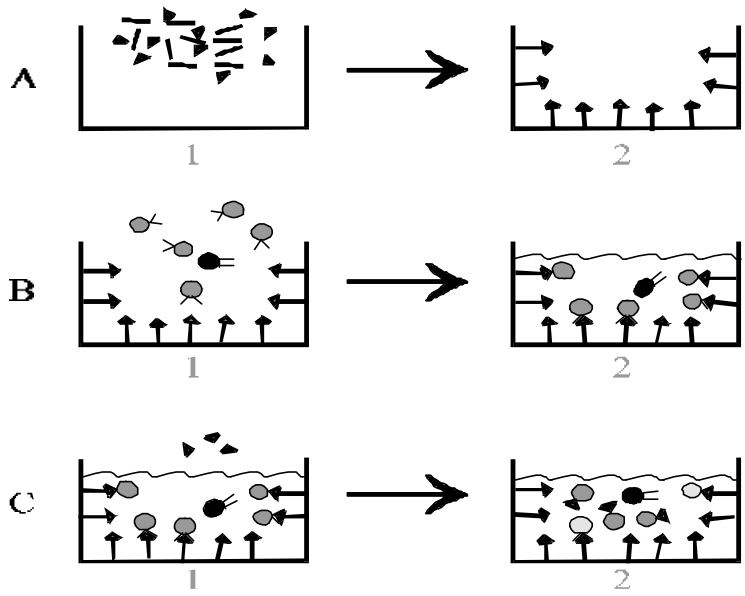


Figure 5.1 Principle of the radioreceptor assay with immobilized ligand-containing microtiter plates. In step A, the ligands (triangles) are immobilized via spacer arms (rods) to the solid phase. In step B, proteoliposomes containing the benzodiazepine receptor (shaded circles) are added to the wells (1) and are allowed to bind to the immobilized ligands (2). Proteoliposomes without the benzodiazepine receptor (but possibly containing other receptors or membrane proteins) are shown as black circles. Step C, shows the addition of free (labeled) ligand (1), which competes with the immobilized ligand for the same receptor binding plate (2). The amount of receptors ultimately bound to the immobilized ligands (light gray circles) depends on the concentration of free ligand added: Increasing the concentration of free ligand is resulting in a decrease of the amount of receptors bound to the immobilized ligand.

Note that this concept is completely different compared to the traditional radioreceptor assay, where the receptor concentration and the amount of labeled ligand in the sample is kept constant, and addition of analyte will ultimately (at higher concentrations of analyte ligand added) lead to saturation of the receptor. The receptor assay described in this Chapter, starts as a normal receptor assay, where the amount of analyte, in this case the ligand immobilized to the wells of the microtiter plates is assumed to be constant, as is the receptor concentration, while the amount of labeled ligand varies. After withdrawal of part of the samples, an additional (second) radioreceptor assay is carried out, where the amount of labeled ligand is constant (and no analyte is present), but in which the receptor concentration is varying, depending on the extent of displacement it has undergone in the first assay. In other words, when a high concentration of labeled ligand is present in the well, the majority of receptors (incorporated in liposomes) are in solution and the highest amount of binding of the labeled ligand to the receptors will be found in de second receptor assay, and vice versa. Therefore, the use of [^{3}H]flunitrazepam, used as labeled ligand, in this case merely serves to demonstrate the principle of the assay, since the calculation of binding constants, as normally done in the traditional assay, is not possible, due to the different set-up.

5.2 Materials and Methods

Materials

Bovine serum albumin (type 5, 96-99%), bovine globulins (Cohn fractions II and III), benzamidine hydrochloride (97%), bacitracin (50,000 units/g), adipic acid dihydrazide, a 240 mg/ml stock-solution of 1-ethyl-3- (3-dimethylaminopropyl)-carbodiimide hydrochloride, sodium iodoacetate, N,N-

dimethyl-formamide, 2-aminoethanol, cholic acid (98%) and sodium deoxycholate (>95%) were obtained from Sigma (St. Louis, MO, USA). Reacti-Bind™ maleic anhydride activated polystyrene 96-well microtiter plates were from Pierce (Rockford, IL, USA). Soybean lipids (asolectin, i.e. crude soybean extract containing approx. 20% PC) were from Avanti Polar Lipids (Alabaster, AL, USA). [*N-methyl*-^{3}H]flunitrazepam (82.0 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Lorazepam was kindly provided by Wyeth Laboratoria (Hoofddorp, The Netherlands). Polyethylene glycol (PEG) 6000 was supplied by Genfarma (Maarssen, The Netherlands), Sephadex™ G-50 Medium by Pharmacia Biotech (Uppsala, Sweden), polyethylene tubes by Greiner (Alphen a/d Rijn, The Netherlands), and glass fiber filters (GF/B) by Whatman (Maidstone, UK). Rialuma scintillation cocktail was obtained from Lumac (Olen, Belgium). Other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Preparation of the microtiter plate containing immobilized benzodiazepine

The coupling of the ligand to the microtiter plate was adapted from a protocol used by Sigel *et al.* (1982) for the preparation of an affinity column for the purification of the benzodiazepine receptor. In short, 200 µl 0.5 *M* adipic acid dihydrazide (pH 7.5) was added to each well of a Reacti-Bind™ maleic anhydride activated polystyrene 96-wells microtiter plate and incubated for 48 h at 35°C. The plate was washed twice by rinsing the wells with distilled water. Incubating the wells with 200 µl 1 *M* 2-aminoethanol (pH 7.5) for 1 h at 35°C blocked excess reactive sites on the plate. The plate was washed with distilled water, 1 *M* NaCl and, again, distilled water. Each well was incubated with 200 µl 30 m*M* sodium iodoacetate (pH 5.0) and 50 µl 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride solution (pH 5.0) for 3 h at 35°C, followed by washing twice with distilled water. To all wells 100 µl 50

m*M* sodium bicarbonate (pH 8.0) and 100 µl N,N-dimethylformamide, containing 0.1 mg/ml didesethylflurazepam (Ro-7-1986) were added, and the plate was incubated for 48 h at 35°C. Excess reactive linker sites were blocked for 1 h at 35°C with 200 µl 1 *M* 2-aminoethanol (pH 7.5) After washing three times with distilled water the plates were used immediately.

Preparation of brain membranes and solubilization of the benzodiazepine receptor

The preparation of a calf brain membrane homogenate and the solubilization of these membranes were done as described by Viel *et al.* (1997). Shortly, calf brains (minus the cerebellum), obtained from the slaughterhouse, were stored at –80°C. The material was thawed and homogenized in 5 volumes (v/w) of ice-cold 0.32 *M* sucrose using a glass-PTFE Potter-Elvehjem homogenizer (R.W.18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was carefully decanted and centrifuged for 60 min at 100,000 *g* at 4°C. The pellet was washed twice with 50 m*M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA followed by centrifugation for 30 min at 100,000 *g* at 4°C. The final pellet was suspended in 5 volumes of the same buffer, rapidly frozen in liquid nitrogen and lyophilized for 48 h.

Lyophilized membranes were suspended to a protein concentration of 8 mg/ml in ice-cold 50 m*M* Tris-HCl (pH 7.4, 22°C) supplemented with protease inhibitors (1 m*M* benzamidine and 200 µg/ml bacitracin), 0.5 *M* KCl and 1 m*M* EDTA and were solubilized on ice by dropwise addition of 20 mg/ml deoxycholate (pH 7.6), to a final detergent concentration of 2 mg/ml. After 30 min, non-solubilized material was removed by centrifugation at 100,000 *g* for 1 h at 4°C. The amount of protein in the solubilized preparation was approximately 5 mg/ml.

Reconstitution of the solubilized benzodiazepine receptor into proteoliposomes by size-exclusion chromatographic detergent depletion.

The procedure for the reconstitution of the solubilized benzodiazepine receptor was done as described by Viel *et al.* (1997). Briefly, soybean lipids (asolectin) were dissolved in chloroform and subjected to rotary evaporation in a round-bottomed flask. Dissolution and evaporation was repeated twice with diethylether and the lipid film was flushed with nitrogen. The lipids were dispersed with 125 m*M* sodium cholate in 50 m*M* Tris-HCl (pH 8.0, 22°C) to obtain a lipid concentration of 75 mg/ml (approximately 100 m*M*). A 1.5 ml aliquot of the lipid solution was mixed with 2.5 ml of deoxycholate-solubilized membranes and applied at 1.5 ml/min at 4°C on a 39 cm × 2 cm Sephadex G-50 M gel bed in 50 m*M* Tris-HCl supplemented with 1 m*M* EDTA and 0.1 *M* KCl (pH 7.6, 22°C). The void volume fractions containing the proteoliposomes were collected and pooled. About half of the total solubilized protein added was recovered in the proteoliposome suspension.

Protein determination

The amount of protein in the receptor preparations was determined by a modified Lowry method (Clark, 1984) after protein precipitation by the addition of trichloroacetic acid to avoid interference of detergent and Tris (Bensadoun and Weinstein, 1976). Bovine serum albumin was used as a standard and was treated as other samples.

Saturation binding experiment

Samples of the proteoliposome suspension [containing 80 µg protein in 180 µl 50 m*M* Tris-HCl supplemented with 1 m*M* EDTA and 0.1 *M* KCl (pH 7.6, 22°C)], were added to the wells of the immobilized benzodiazepine microtiter plate and were incubated for 2 h at 0-2°C with 20 µl aliquots of

[^{3}H]flunitrazepam stock solutions (in 50 m*M* Tris-HCl (pH 7.6, 22°C) supplemented with 1 m*M* EDTA) giving final concentrations of 0.1-10 n*M* [^{3}H]flunitrazepam. The total incubation volume was 200 µl. After incubation, 150 µl aliquots of the samples were transferred from the wells into polyethylene tubes, the concentration of [^{3}H]flunitrazepam in all samples was adjusted to 10 m*M*. These samples were incubated further for 45 min at 0-2°C. After the latter incubation, 15 µl of 33 mg/ml bovine globulins and 85 µl of a 360 mg/ml PEG-6000 solution were added. The incubation was continued for another 12 min and then stopped by the addition of 3 ml of ice-cold 50 m*M* Tris-HCl buffer (pH 7.6, 22°C) supplemented with 1 m*M* EDTA, after which the samples were immediately filtered through Whatman GF/B glass fiber filters under vacuum using a filtration apparatus (48S, University Centre for Pharmacy, Groningen, The Netherlands). The tubes were rinsed twice with 3 ml portions of the same buffer, which were also filtered. The total filtration and rinsing process of each separate tube was done within 15 s. The filters were then transferred to scintillation vials and dispersed in 3.5 ml scintillation cocktail by shaking for 2 h. The vials were counted for 40,000 counts or 5 min, whatever came first, in a liquid scintillation counter (Minaxi, Packard, Groningen, The Netherlands).

5.3 Results and Discussion

A benzodiazepine (didesethylflurazepam or Ro7-1986) was immobilized on a maleic anhydride-activated polystyrene 96-wells microtiter plate via a hydrophilic spacer of 15-atom length, three of which are originating from the benzodiazepine itself (Figure 5.2). The procedure followed is essentially as described by Sigel *et al.* (1982) for immobilizing didesethylflurazepam to

agarose for affinity chromatographic purification of the benzodiazepine receptor. Three major steps were involved in the immobilization procedure:

1. coupling of adipic acid dihydrazide (AAD) to the maleic anhydride-activated microtiter plate
2. reaction between immobilized AAD and iodoacetate (using the carbodiimide as a catalyst)
3. coupling of the benzodiazepine to the formed spacer

Didesethylflurazepam has a primary amino function on the N_1 position that is not involved in the interaction with the receptor. Besides, coupling of this benzodiazepine via a spacer arm to a solid phase essentially does not alter its affinity towards the receptor. Therefore, this ligand is suitable for immobilization procedures. The length of the spacer arm is another critical parameter. Since the binding site of a receptor is located inside the membrane protein, the use of a short spacer arm (approximately 1-7 atoms length) or no spacer at all, will yield a ligand immobilized such, that it can not reach the binding site. The length of 15 atoms was chosen by Sigel *et al.* (1982) and appeared to work properly for immobilization to an agarose column for purification of the benzodiazepine receptor and, therefore, we chose this length for our experiments.

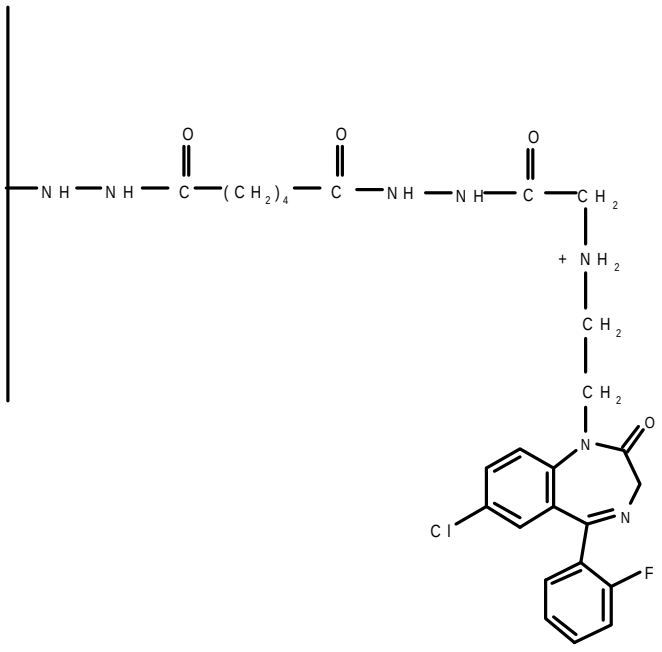


Figure 5.2 Structure of didesethylflurazepam (Ro7-1986) immobilized via a 15-atom length spacer to a solid support (maleic anhydride activated polystyrene 96-well microtiter plate).

The main difference between the immobilization procedure as described by Sigel and the one described here is the reaction temperature. All reactions were done at 35°C instead of room temperature. At room temperature, almost no coupling of the benzodiazepine via the spacer to the microtiter plate was observed.

Didesethylflurazepam in the concentration range $5x10^{-6}$ to 0.05 mg/ml (final concentrations) was added to the (reactive) immobilized spacer. It was found that 0.05 mg/ml was a suitable concentration to immobilize. When lower concentrations were used, the amount of receptors that bound to the immobilized ligand was too low, as determined with the radioreceptor assay (results not shown). Higher concentrations were considered impractical, since only a small amount of the ligand was available to our research group.

From the 3-step immobilization procedure, only the first step (the coupling of AAD to the microtiter plate) could be (qualitatively) checked, with 2,4,6-trinitrobenzenesulfonate (TNBS). The latter reacts with a primary amino group, giving a yellow color.

Since no antibodies against didesethylflurazepam were available, immobilization of this ligand via its spacer to the microtiter plate could only be checked indirectly with a radioreceptor assay. However, because the ligand was immobilized, an alternative radioreceptor assay had to be developed: proteoliposomes were bound to the immobilized ligand and, when [^{3}H]flunitrazepam was added, there was competition between the radiolabeled ligand and the immobilized ligand for the receptors. Hence, increasing

concentrations of the radiolabeled ligand would remove receptors from the immobilized ligand back into the solution. As a result, a higher receptor concentration can be found in the samples containing higher concentrations of [^{3}H]flunitrazepam. After incubation at 0°C for 2 h, 150 µl samples were transferred from the wells to a polyethylene tube, and the concentration of [^{3}H]flunitrazepam in all samples was adjusted to 10 m*M* to provide a large excess, thus making sure that a constant percentage (>80%) of the receptors in the solution are radioactively labeled. The labeled receptors were then filtered off on glass fiber filters and the radioactivity on the latter was counted. Thus, in this type of receptor assay the amount of receptor in the sample solution is determined. Figure 5.3 shows the results of the above described receptor assay. The IC_{50}, in this case the amount of labeled ligand that could displace 50% of the receptors bound to the immobilized ligand, can be expressed as:

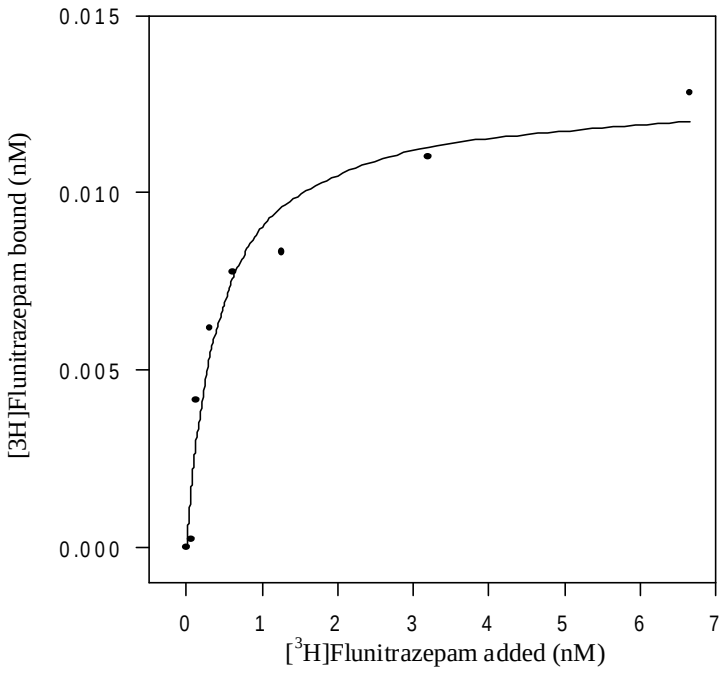
$$IC_{50} = K_{d(flunitrazepam)} * \left(1 + \frac{[F_{(immobilized)}]}{K_{d(immobilized)}} \right)$$

Since the concentration of free, immobilized, ligand ($F_{(immobilized)}$) is approaching 0 (in the microtiter plate only a very small layer near the solid phase contains this ligand, while in the bulk solution (and in the samples transferred to polyethylene tubes for the further incubation) this ligand is absent), the IC_{50} should equal the value of the K_d of flunitrazepam. As seen in Chapter 3, the K_d of flunitrazepam was 2.8 n*M* for benzodiazepine receptors reconstituted in asolectin proteoliposomes, whereas it can be estimated from Figure 5.3 to be approximately 0.5 n*M*.

Since this receptor assay is different from the normal assay, it is not possible to (accurately) calculate all receptor binding constants. For the latter, the receptor density has to be constant. Therefore, this is not a quantitative receptor assay. It

only shows that the principle of a receptor assay based upon immobilized ligand binding to receptors incorporated in liposomes works properly.

Procedures for the reconstitution of other receptors and immobilization protocols have been described in the literature (see Chapter 3). It can be expected that the above assay set-up can also be used for other receptors than



the benzodiazepine receptor.

Figure 5.3 Radioreceptor assay after a competition between ligand immobilized to a microtiter plate and radioligand ([^{3}H]flunitrazepam) for benzodiazepine receptors. See text for details. Results are the means of two separate experiments.

Based on the above observations, a quantitative receptor assay may be developed using fluorescent labeled proteoliposomes, in which the amount of

receptors bound to the solid phase-immobilized ligand can be measured directly and more accurately (see Chapter 6).

5.4 Conclusions

Immobilization of a benzodiazepine (didesethylflurazepam) to a microtiter plate can be used to develop a solid phase receptor assay, in which the receptor is incorporated in liposomes. The immobilization procedure could be easily adapted from the protocols available for immobilization of benzodiazepine ligands on an affinity column for chromatographic purification of the receptor.
Competition between the added (labeled) ligand and the immobilized ligand for the receptor binding site, takes place in a small volume (the area near the walls of the microtiter plate). As a result, the sensitivity of the assay is higher, since the concentration of free, immobilized ligand does not play a role in the calculation of the IC_{50} value. This leads to lower values of the IC_{50} compared to the traditional radioreceptor assay. The receptors were incorporated into proteoliposomes, since the latter can be easily labeled with fluorescent lipids (Chapter 4) for the development of a non-radioactive receptor assay (see Chapter 6), but the assay should work equally well when membrane suspensions (crude homogenate) are used.

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Chapter 6

A Novel Fluorescent Benzodiazepine Receptor Assay Using Fluorescent Labeled Proteoliposomes and a Ligand Immobilized in Microtiter Plates

6.1 Introduction

Receptor assays are routinely used for the assessment of the interaction of (new) chemical entities with a given receptor as well as for the quantitation of drugs that exert their action via a particular receptor. Due to the fact that in general the binding of a compound to a receptor cannot be quantitated directly, labeled ligands with high affinity towards the receptor are being used. Competition between the labeled ligand and the analyte for the same receptor binding site will take place in the sample and can be monitored by quantitation of either the free or the receptor- bound fraction of the labeled ligand. Most labeled ligands used in receptor assays are radioligands. The major advantage of using radioligands (mainly tritium and iodine labeled ligands) in receptor assays is that the spatial structure of the ligand is not or only slightly modified by the incorporation of an isotope. Therefore, the affinity of the labeled ligand can be expected to be comparable or even identical to that of the unlabeled compound. Besides, radioligands are chemically stable and can be detected with a high sensitivity and over a wide concentration range. However, there are several disadvantages in using radiolabels, including high costs, radioactive waste, health hazards, and the need of special equipment and licenses. For these reasons fluorescent labels for the use in receptor assays have been developed, as described for the α- and β-adrenergic and the opiate receptor by Corrêa *et*

al., (1980), the benzodiazepine receptor (Takeuchi and Rechnitz, 1991, and Janssen, 1997) and the estrogen receptor (Hwang *et al.*, 1992). Because most high affinity ligands available do not have native fluorescence, fluorescent functionalities are to be coupled directly or via a (chemical) spacer-arm to the ligand. However, the attachment of a (bulky) fluorescent moiety to a ligand often results in diminished affinity of the ligand to its receptor. Moreover, since ligands are bound inside the receptor protein, many positions on the ligand are unsuitable for the attachment of fluorescent labels. For example, coupling of fluorophores to 1,4-benzodiazepines and 1,2-annelated analogs, either at the 3- or 7-position (Figure 6.1), resulted in a substantial decrease in affinity of that benzodiazepine towards its receptor, whereas labeling at the 1-position may yield a similar or slightly lowered affinity (Janssen, 1997).

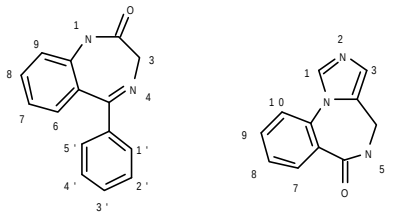

Figure 6.1 Basic structures of 1,4-benzodiazepines (left) and 1,2-annelated 1,4-benzodiazepines (right).

The development of high affinity fluorescent labeled ligands is merely a matter of trial and error, because hardly any relevant Structure Activity Relationships are available for ligands containing bulky groups. Furthermore, background fluorescence caused by the proteins present in the receptor preparation may overshadow the changes in fluorescence that can be ascribed to changes in the bound or free fraction of the labeled ligand. Therefore, it would be preferable to avoid labeling of ligands and design alternative (fluorescent) receptor assays.

This Chapter describes the development of such a novel fluorescent benzodiazepine receptor assay. Receptors incorporated in fluorescent liposomes (see Chapter 4) can be bound to a benzodiazepine immobilized to a microtiter plate (as already described in Chapter 5). Competition with analyte present in the sample will lead to a decrease in the amount of receptor bound to the immobilized ligand and, hence, in a decrease in the amount of fluorescence that can be detected after washing of the plate. Figure 6.2 shows a schematic presentation of the method.

Instead of labeling the ligands, the receptors are (indirectly) labeled in this method. Since there is no need to label the ligands with bulky fluorescent moieties, we expect that no dramatic decrease in affinity will be seen, although the immobilized ligand might have a somewhat lower affinity towards the receptor. Because many fluorescent lipids are present per single receptor, low receptor concentrations can be used and quantitated with high precision. Theoretically, this might improve the limits of detection and quantitation.

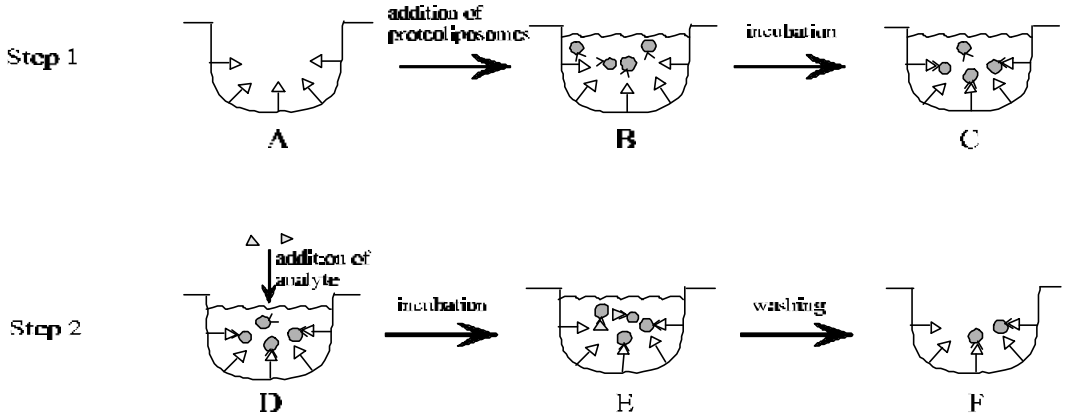

Figure 6.2 Principle of the fluorescent receptor assay, where receptors incorporated in fluorescent labeled liposomes compete between ligand immobilized on a microtiter plate and the analyte present in the sample. Step 1: to a microtiter plate containing immobilized ligands (A), proteoliposomes are added (B) and incubated under proper conditions, until most receptors are bound to the immobilized ligand in the wells (C). In step 2, competition with analyte present in the sample that is being added (D) leads to an equilibrium situation, where part of the receptor-containing proteoliposomes are bound to the immobilized ligand, and part of them are bound to the analyte (E). After

washing, fluorescence can be determined in the well and is a (reversed) measurement for the amount of analyte that was present in the sample (F).

6.2 Materials and Methods

Materials

Bovine serum albumin (type 5, 96-99%), bovine globulins (Cohn fractions II and III), benzamidine hydrochloride (97%), bacitracin (50,000 units/g), adipic acid dihydrazide, 1-ethyl-3- (3-dimethylaminopropyl)carbodiimide hydrochloride, sodium iodoacetate, N,N-dimetyl-formamide, 2-aminoethanol, cholic acid (98%) and sodium deoxycholate (>95%) were obtained from Sigma (St. Louis, MO, USA). Reacti-Bind™ maleic anhydride activated polystyrene 96-well microtiter plates were from Pierce (Rockford, IL, USA). Soybean lipids (asolectin, i.e. crude soybean extract containing approximately 20% phosphatidyl choline) were from Avanti Polar Lipids (Alabaster, AL, USA), N-(5-fluoresceinthiocarbamoyl)-1,2-dihexa-decanoyl-sn-glycero-3-phosphoethanolamine, triethyl-ammonium salt (fluorescein DHPE) was from Molecular Probes, Inc. (Eugene, OR, USA). Lorazepam was kindly provided by Wyeth Laboratoria (Hoofddorp, The Netherlands). Sephadex™ G-50 Medium was supplied by Pharmacia Biotech (Uppsala, Sweden). Other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Preparation of microtiter plates containing immobilized benzodiazepine

Coupling of the ligand to the microtiter plate was done essentially as described by Sigel *et al.* (1982) and Taguchi and Kuriyama (1984) for the preparation of an affinity column for the purification of benzodiazepine receptors with some minor modifications (see Chapter 5). In short, to each well of a Reacti-Bind™

maleic anhydride activated polystyrene 96-well microtiter plate, 200 µl 0.5 *M* adipic acid dihydrazide (pH 7.5) was added and incubated for 48 h at 35°C. The plate was washed twice by rinsing the wells with distilled water. Incubating the wells with 200 µl 1 *M* 2-aminoethanol (pH 7.5) for 1 h at 35°C blocked excess reactive sites on the plate. The plate was washed with distilled water, 1 *M* NaCl and, again, distilled water. Each well was incubated with 200 µl 30 m*M* sodium iodoacetate (pH 5.0) and 50 µl (from a 240 mg/ml stock solution of) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (pH 5.0) for 3 h at 35°C, followed by washing twice with distilled water. To all wells 100 µl 50 m*M* sodium bicarbonate (pH 8.0) and 100 µl N,N-dimethylformamide, containing 0.1 mg/ml didesethylflurazepam (Ro-7-1986) were added, and the plate was incubated for 48 h at 35°C. The plates were subsequently incubated for 1 h at 35°C with 200 µl 1 *M* 2-aminoethanol (pH 7.5) in order to block the excess of reactive linker sites. After washing three times with distilled water, the plates were used immediately.

Preparation of brain membranes and solubilization of the benzodiazepine receptor

The preparation of a calf brain membrane homogenate and the solubilization of these membranes were done as described by Viel *et al.* (1997). Shortly, calf brains (minus the cerebellum), obtained from the slaughterhouse, were stored at –80°C. The material was thawed and homogenized in 5 volumes (v/w) of ice-cold 0.32 *M* sucrose using a glass-PTFE Potter-Elvehjem homogenizer (R.W.18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was carefully decanted and centrifuged for 60 min at 100,000 *g* at 4°C. The pellet was washed twice with 50 m*M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA followed by centrifugation for 30 min at 100,000 *g* at 4°C. The final

pellet was suspended in 5 volumes of the same buffer, rapidly frozen in liquid nitrogen and lyophilized for 48 h.

Lyophilized membranes were suspended to a protein concentration of 8 mg/ml in ice-cold 50 m*M* Tris-HCl (pH 7.4, 22°C) supplemented with protease inhibitors (1 m*M* benzamidine and 200 µg/ml bacitracin), 0.5 *M* KCl and 1 m*M* EDTA and were solubilized on ice by dropwise addition of 20 mg/ml deoxycholate (pH 7.6), to a final detergent concentration of 2 mg/ml. After 30 min, non-solubilized material was removed by centrifugation at 100,000 *g* for 1 h at 4°C. The amount of protein in the solubilized preparation was approximately 5 mg/ml.

Reconstitution of the solubilized receptor into fluorescent proteoliposomes, by size-exclusion chromatographic detergent depletion.

Reconstitution of the benzodiazepine receptor in (fluorescent) asolectin liposomes was done as described by Viel *et al.* (1997). In short, 150 mg soybean lipids (asolectin) and 250 µg fluorescein-DHPE (used as fluorescent lipid probe) were dissolved in chloroform and subjected to rotary evaporation in a round-bottomed flask. Dissolution and evaporation was repeated twice with diethylether and the lipid film was flushed with nitrogen. The lipids were dispersed with 125 m*M* sodium cholate in 50 m*M* Tris-HCl (pH 8.0, 22°C) to obtain a lipid concentration of 75 mg/ml (approximately 100 m*M*). A 1.5 ml aliquot of the lipid solution was mixed with 2.5 ml of deoxycholate-solubilized membranes and applied at 1.5 ml/min at 4°C on a 39 cm × 2 cm Sephadex G-50 M gel bed in 50 m*M* Tris-HCl supplemented with 1 m*M* EDTA and 0.1 *M* KCl. The void volume fractions, containing the proteoliposomes, were collected and pooled (approximately 15 ml total), and were used immediately for further experiments (alternatively, the proteoliposomes could be stored at

–20°C or –80°C, as described in Chapter 3). About half of the total solubilized protein was recovered in the proteoliposome suspension.

Protein determination

The amount of protein in the receptor preparations was determined by a modified Lowry method (Clark, 1984) after protein precipitation by the addition of trichloroacetic acid to avoid interference of detergent and Tris (Bensadoun and Weinstein, 1976). Bovine serum albumin was used as standard and was treated as other samples.

Binding experiments

Samples of the fluorescent-labeled proteoliposome suspension [containing 80 µg protein in 180 µl 50 m*M* Tris-HCl supplemented with 1 m*M* EDTA and 0.1 *M* KCl (pH 7.6, 22°C)], were added to the wells of the microtiter plate with immobilized benzodiazepine and were incubated for 2 h at 4°C with 20 µl aliquots of lorazepam stock solutions (in 50 m*M* Tris-HCl (pH 7.6, 22°C) supplemented with 1 m*M* EDTA) giving final lorazepam concentrations of 300 n*M*-30 p*M*. The total volume was 200 µl. After this incubation-period, the plate was washed three times with ice-cold Tris/EDTA buffer and the bound (fluorescent) proteoliposomes were measured in a BioTek FL500 microplate fluorescent reader (Winooski, VT, USA), using an excitation wavelength of 496 nm and an emission wavelength of 519 nm.

6.3 Results and Discussion

Principle of the fluorescent receptor assay

The principle of this alternative fluorescent receptor assay, using a microtiter plate to which didesethylflurazepam was immobilized, is depicted in Figure 6.2.

Benzodiazepine receptors reconstituted in asolectin liposomes also containing fluorescein-DHPE as the fluorescent label (Chapter 4) were bound to benzodiazepine ligands (didesethylflurazepam) immobilized on a microtiter plate (Chapter 5). The incubation time was prolonged (2 h total) because the ligand may not have been homogeneously distributed, therefore the association time of the binding of (immobilized) ligand to its receptor might be longer than in a homogeneous receptor binding assay. Further experiments are required to confirm this. Competition with analyte (lorazepam) present in the incubation mixture led to a reduction in binding of the receptor to the immobilized ligand, depending on the concentration of analyte present in the sample. An increase in the concentration of the analyte causes a proportional decrease in the amount of receptors bound to the immobilized ligand. Since the receptors are incorporated in fluorescent-labeled liposomes, the amount of fluorescence that can be detected after washing the plate, is an inverse measure of the concentration of analyte present in the sample.

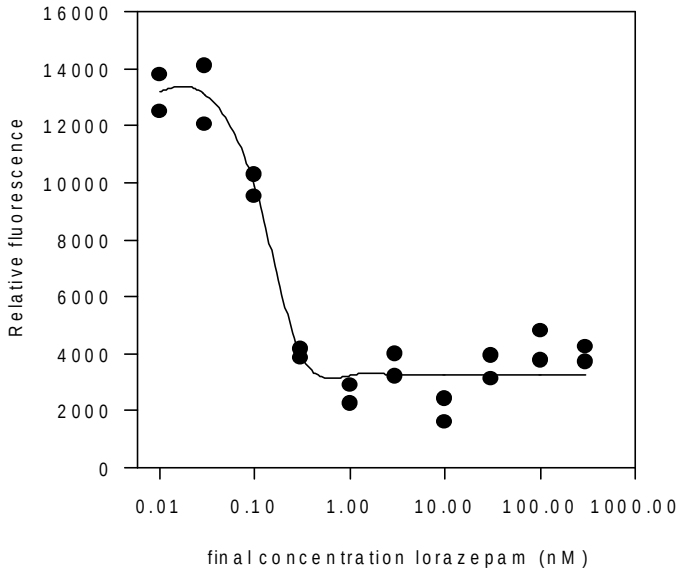

Figure 6.3 Inhibition curve: Fluorescent proteoliposomes containing the benzodiazepine receptor were incubated with a fixed amount of immobilized ligand in the presence of increasing concentrations of a competitive ligand, lorazepam. The results of two separate experiments are presented in the figure.

Binding experiments

Proteoliposomes containing the benzodiazepine receptor and fluorescein DHPE were added together with 300 n*M*-30 p*M* lorazepam (which was used as the analyte) to the wells of an immobilized didesethylflurazepam microtiter plate. After a 2 h incubation period at 4°C the wells were washed three times with ice-cold 50 m*M* Tris-HCl supplemented with 1 m*M* EDTA and the amount of fluorescence was measured on a fluorescence plate reader. The inhibition curve is shown in Figure 6.3.

The IC_{50}-value for lorazepam as determined with this assay is estimated as approximately 0.2 n*M*, which is considerably lower than the values determined by Janssen (1997) for a fluorescent receptor assay (FRA) or for the radioreceptor assay (RRA), which were 7.2±0.5 n*M* and 6.6±0.7 n*M*, respectively, which is approximately a factoe 30. A possible explanation is that the association binding constant of immobilized ligand is (much) lower or the dissociation binding constant of immobilized ligand is (much) higher than the binding constants of lorazepam in solution. To check these possibilities, the amount of didesethylflurazepam immobilized in the wells of the microtiter plate and the association and dissociation binding kinetics of immobilized didesethylflurazepam to the benzodiazepine receptor have to be determined in more detail. However, as seen in Chapter 5, the saturation curve of [^{3}H]flunitrazepam in the microtiter plate, also showed a lowering in the IC_{50}-value. Due to the set-up of the experiment, in which competition between immobilized ligand and analyte only takes place in the vicinity of the walls of the wells, an increase in sensitivity can be anticipated. The factor 30 gain in sensitivity found, therefore, might be caused by an increase in affinity of the receptor on the one hand, and an additional increase in sensitivity caused by amplification of the fluorescence signal, due to the presence of several fluorescent lipids per single receptor protein. As a consequence, the detection limit for benzodiazepines is lowered.

The nonspecific binding of fluorescent proteoliposomes (determined as the amount of fluorescence present (after washing) in the plates incubated with an excess concentration of lorazepam) was approximately 25%, which is comparable to fluorescent receptor assays utilizing fluorescent ligands (Janssen, 1997, Takeuchi *et al.*, 1991 and 1993).

6.4 Conclusions

The benzodiazepine receptor assay could be performed successfully in a modified version, in that the ligand (didesethylflurazepam) was immobilized in the wells of a microtiter plate and the receptor was present in the aqueous medium, incorporated in fluorescent proteoliposomes. When an aliquot containing the analyte was added, the latter competed with the immobilized ligand in the binding with the receptors in the proteoliposomes. Further experiments are needed to optimize the assay and to validate it. When the amount of ligand immobilized in the wells is determined and optimized, the amount of receptors can be chosen so that the accuracy and sensitivity are optimal. However, at this time, already an increase in sensitivity of a factor 30 has been found, compared to receptor assays using a fluorescent ligand. The time to perform the immobilized ligand receptor assay is still longer than in the conventional assay, due to the prolonged incubation time. Further kinetic studies should reveal if the incubation period could be shortened. Nonspecific binding was comparable to fluorescent receptor assays using fluorescent-labeled ligands, and might be reduced when a more purified receptor material is used for the preparation of the proteoliposomes. Also, the walls of the microtiter plate may be coated to prevent nonspecific binding of proteoliposomes to the solid phase.

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Chapter 7

General Conclusions and Perspectives

In this thesis the development of a novel fluorescent receptor assay is described. Thus far, most attempts to develop a nonradioactive receptor assay were focused on the labeling of the ligand of choice by coupling a (large) detectable moiety to it. Since various positions on the ligand are involved in the actual receptor binding, more often than not this approach leads to reduction or elimination of the affinity of that ligand towards its receptor. For that reason, the selection of a suitable ligand and the construction of a labeled ligand with high affinity can be laborious and the expenses might be high.

Therefore, our goal was to construct a receptor assay, in which there was no need to label the ligand. Instead, we set out to label the receptor. To do this, we immobilized a suitable benzodiazepine ligand (didesethylflurazepam) via a hydrophilic spacer of 15-atom length (three of which originate from the benzodiazepine molecule) to a maleic anhydride-activated polystyrene microtiter plate. A brain membrane homogenate was solubilized with sodium deoxycholate and the benzodiazepine receptors were reconstituted together with fluorescent lipids in asolectin liposomes. The resulting proteoliposomes, containing the benzodiazepine receptor and analyte (lorazepam in increasing concentrations) were added to the wells of the microtiter plate. The analyte and the immobilized ligand compete for binding to the receptor, incorporated in the labeled liposome. Increasing concentrations of analyte in the sample reduced the amount of fluorescent liposome-receptor complex bound to the immobilized ligand in the well. After removing the supernatant, the

fluorescence in the well was found to be inversely related to the concentration of analyte in solution.

Binding of the labeled proteoliposomes containing the benzodiazepine receptor to the immobilized ligand can be described as:

$$L_I + R^* \leftrightarrow L_IR^* \qquad (1)$$

L_I = immobilized ligand

R^* = proteoliposomes containing the receptor and the fluorophore

L_IR^* = fraction of labeled proteoliposomes bound to the immobilized ligand

When an analyte is added the following equation describes the process:

$$L_I + R^* + A \leftrightarrow L_IR^* + AR^* \qquad (2)$$

A = analyte

AR^* = fraction of labeled proteoliposomes bound to the analyte, and present in the supernatant

If a labeled proteoliposome does not contain a receptor it will not bind to the immobilized ligand. Probably the number of liposomes that contain receptor proteins is relatively small, considering the size of the receptor protein ($M_r \approx$ 300,000, or approximately 5 nm) in comparison with the average size of the liposomes (approximately 75 nm).

In this approach the fraction L_IR^* in (2) was monitored, the amount of this fraction is inversely related to the concentration of analyte. Ensing *et al.* [1,2] compared the accuracy and precision profiles for radioreceptor assays in which either the bound or the free fraction of labeled ligand were quantitated and related to the concentration of analyte. Provided that a substantial part of the

labeled ligand (>50%) was receptor-bound in the absence of analyte, measurement of the free fraction of labeled ligand in the supernatant, collected after a centrifugation step, improves the precision for low analyte concentrations. For higher analyte concentrations, measurement of the bound fraction labeled ligand, collected after a filtration step, is the preferred option. If the situation should occur in which each proteoliposome contains at least 1 active receptor protein (and, assuming that the there is an excess immobilized ligand capable of binding the majority of the receptor proteins), measurement of the fraction labeled proteoliposomes, present in free form or complexed with the analyte, is a viable option for this type of receptor assay. However, since a considerable proportion of the proteoliposomes has relatively small sizes, as determined in Chapter 3, they simply cannot contain the large benzodiazepine receptor complex, therefore causing a high background fluorescence signal in the free fraction. Besides, it can be estimated (assuming an average diameter for the proteoliposomes of 75 nm) that 1 receptor protein per 750 proteoliposomes is present. It is therefore preferred to measure the fraction of receptors bound to the immobilized ligand in this assay.

Immobilization of ligands

Methods have been developed for the preparation of columns for the affinity chromatographic purification of receptors, which could be easily adapted for the immobilization of ligands to solid phases. In our case, a benzodiazepine was immobilized to a maleic anhydride-activated polystyrene microtiter plate (see Chapter 5). Because such (affinity chromatographic) protocols are also available for the purification of other receptors (examples can be found in Refs. 3-7), it can be expected that microtiter plates with suitable immobilized ligands can also be developed for a variety of other receptors.

One drawback of the present immobilization method is that it is not easy to determine the amount of ligand that is bound to the plate after immobilization without the availability of e.g. antibodies directed against that ligand. In our case, we do not actually know how much ligand was immobilized per well, but we immobilized increasing concentrations of ligand and selected that concentration that gave the highest amount of specific binding (total binding – non-specific binding) to the benzodiazepine receptor, as measured in the receptor assay. In principle, ligands with the highest affinity towards the receptor are preferable for immobilization. For example, the benzodiazepine ligand 1012-S has a very high affinity for the benzodiazepine receptor and has been used successfully for the development of an affinity column for the purification of the receptor complex. It should be noted, however, that the binding affinity of an immobilized ligand to the receptor might be different from the affinity of the same ligand in solution. Even inactive precursors or metabolites of high affinity compounds may be used, when coupling of the spacer arm introduces functionalities required for high affinity interaction.

Labeled liposomes

Since receptors were found to be rather unstable when solubilized from membrane fragments of the brain homogenate, liposomes were used to provide a more stable embedding environment and to incorporate suitable detection labels. The rationale behind this is to obtain small spherical particles (100-500 nm diameter) with one single or a few receptor proteins, together with a large quantity of detectable functionalities. Since brain membranes do not fulfill this requirement, (partial) purification of the receptor proteins is the next step.

For various membrane proteins, solubilization- and reconstitution procedures have been described (Table 7.1 and Table 7.2). The solubilization of membrane-bound receptors is the most critical step, since the use of detergents,

necessary to separate the proteins from their lipid environment, can easily lead to denaturation of the

Table 7.1 Conditions for solubilization of different types of membrane receptors from animal tissue or cell cultures. [a] : besides detergents protease inhibitors were used. The incubation temperature was 0-4°C except for digitonin (room temperature). For other conditions, see Chapter 2.

receptor	tissue	detergent[a]	recovery of receptor binding	reference
benzodiazepine	bovine cortex	20 m*M* CHAPS	67%	8
benzodiazepine	rat brain cortex	0.7% Triton X-100	60-70%	9
benzodiazepine	bovine cortex	2% desoxycholate	54±6%	13
histamine H-1	guinea pig brain	1% digitonin	40-60%	10
μ-opioid	7215c cells	10 m*M* CHAPS	40±5%	11
muscarinic	bovine cortex	1% digitonin	34%	12

Table 7.2 Reconstitution of membrane receptors in lipid vesicles. [a] : abbreviations used: PC = phosphatidyl choline, Ch = cholesterol, Cholh = cholesteryl hemisuccinate. [b] : SEC = size exclusion chromatography, also known as gel-filtration. [c] : the myometrial oxytocin receptor binding activity after reconstitution was dependent on the addition of cholesterol; without cholesterol receptor binding could not be restored.

receptor	lipids used[a]	method use for detergent depletion	reference
benzodiazepine	asolectin	SEC[b]	13
benzodiazepine	brain lipid extract	SEC[b]	14
myometrial oxytocin	PC/Ch/Cholh[c]	bio-beads SM-2	15
vasopressin	asolectin	SEC[b]	16
opioid	brain lipids	PEG precipitation	17

receptors and therefore impair their capacity to bind pharmacologically active substances. The use of mild detergents and detergent concentrations as low as possible are hence important determinants. We used sodium deoxycholic acid for solubilization of the crude brain membrane homogenate (Chapter 2). Although this detergent is fairly aggressive, since it is ionic, it yielded higher amounts of soluble receptors than other detergents.

Still, even at low concentrations of deoxycholate, a substantial loss of receptor binding was observed after solubilization. This indicated that only a certain amount of the receptor proteins were solubilized and/or that the receptor binding was impaired to some extent.

The reconstitution of the solubilized benzodiazepine receptor into proteoliposomes by size-exclusion chromatography was found to be less critical (Chapter 3). Since it is a fast procedure, the exposure time of the

receptor proteins to detergents was limited to a minimum, in contrast to dialysis. As with many other receptors, the type of lipids used for the reconstitution had little influence on receptor activity although the best results were obtained with a mixture of phospholipids (asolectin).

Solubilization followed by reconstitution of membrane proteins could thus be used to make lipid vesicles that contain the receptor of interest plus a label that allows simple and sensitive detection and quantitation of the receptor-vesicle complex. However, the amount of (active) receptor proteins after solubilization and reconstitution was considerably lower than found in the initial membrane homogenate; i.e. maximally only about 30% of the benzodiazepine receptors could be recovered in liposomes. Purification of the receptor, for instance by affinity chromatography might be used to increase the receptor density in the liposome population. Additional advantages of the latter are that, since other (interfering) proteins are not incorporated, the amount of nonspecific binding consequently will be lowered, and that a higher receptor density in the liposomes may allow further miniaturization of the assay. Ideally, each liposome should contain one active receptor in order to obtain maximal assay sensitivity.

Alternatively, proteoliposomes might be made without prior solubilization of the membrane proteins. Preliminary results with sonication showed that membrane vesicles could be formed directly from crude membrane homogenate. Furthermore, sonication in combination with freezing and thawing, followed by extrusion of the vesicles through membrane filters might yield a more homogeneously sized population of proteoliposomes.

Fluorescent lipids could be co-reconstituted in the above approach, thus forming labeled proteoliposomes (see Chapter 4). In principle, a large variety

of labels can be included in the membranes of the proteoliposomes, including radiolabeled lipids or enzymes (Figure 7.1). Alternatively, the (aqueous) interior of the proteoliposomes can be filled with suitable labels, like chromophores or soluble enzymes. However, one has to take measures to prevent such proteoliposomes from leaking.

Radiolabels, preferably ^{3}H or ^{125}I, attached to e.g. the lipids, remain attractive as a form of labeling, since large numbers of labels per liposome, and therefore, per receptor binding site, can be obtained. Moreover, quantitation of bound and/or free fractions of radiolabeled liposomes is relatively simple and reliable. In addition, considering the availability of multi-channel detectors, high throughput can also be warranted. However, widespread use of a radioactive approach is limited because of legal limitations in the use of radioactive substances and the disposal of radioactive waste. Thus, nonradioactive functionalities remain preferable.

Functionalities that can readily be detected with spectroscopic (preferably fluorescent or chemiluminescent techniques) therefore have a better practical potential. In contrast to traditional receptor assays with fluorescent labeled ligands, the selection of the fluorophore for the labeling of proteoliposomes is not affected by a possible effect on receptor binding properties. Thus, any fluorophore with favorable spectroscopic properties can be used. Favorable spectroscopic properties are an emission wavelength and, preferably, an excitation wavelength of the fluorophore longer than 450 nm in order to limit background fluorescence by membrane proteins that are also reconstituted in the liposomes. We selected a fluorescein-labeled phosphatidylethanolamine (fluorescein-DHPE) as the fluorescent lipid, which was mixed with the lipids used for the reconstitution process (asolectin). As a result the fluorescence label was about 0.2% of the total lipid content. Higher label concentrations yielded higher fluorescence signals but it also introduced some quenching.

A different type of detectable functionality, offering new opportunities for the reduction in sample size and for improving method sensitivity is formed by enzymes. The latter can be incorporated in liposomes containing the receptor of interest and amplify a signal by converting a precursor into a detectable product, preferably of fluorescent or chemiluminescent nature. Examples of such enzymes are monoamine oxidase and alkaline phosphatase. An assay based on luciferase may also have high potential, in that only an increase in signal, and virtually no background signal, is obtained.
However, fluorescent lipids are commercially available for reasonable prices and are easy to incorporate in the liposomes. Detection can be obtained in a single step, in contrast to using enzymes as the detection mode.

A major benefit of the approach presented here is that much smaller amounts of receptors can be used, because a multitude of label (at least 50) is present per single receptor protein and because the area in which receptor binding occurs is restricted to the surface of the wells (Chapter 6). Therefore, miniaturization of the assay is an option, which is particularly beneficial when materials are scarce or expensive. The use of microtiter plates allows automation (of a large part) of the receptor assay.

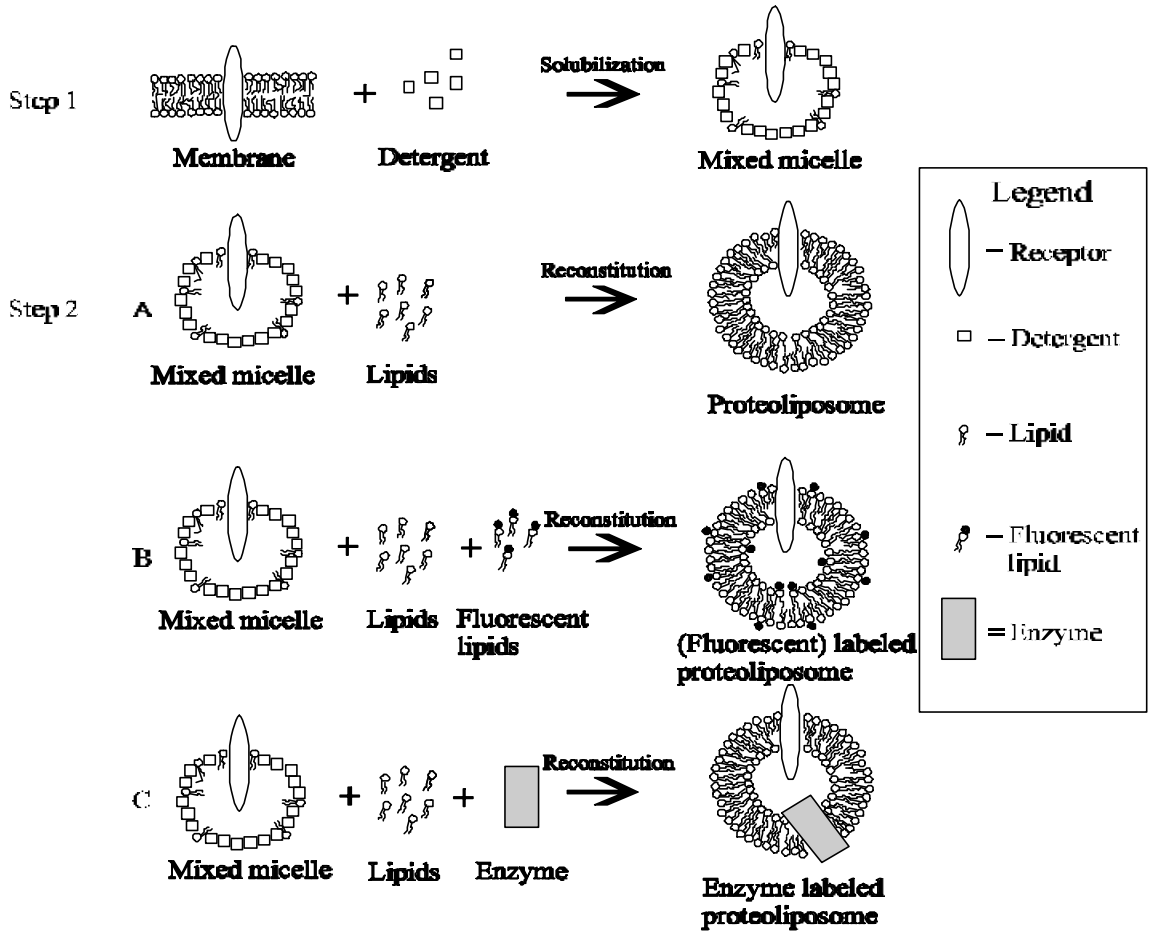

Figure 7.1 Schematic presentation of the solubilization of membrane receptors (Step 1) and their reconstitution in liposomes (Step 2A), together with appropriate detection labels, such as fluorescent lipids (Step 2B) or enzymes (Step 2C).

After removal of the receptor-containing proteoliposomes, the microtiter plates with immobilized ligand can be reused. In Chapter 6 it was shown that the sensitivity, in terms of IC_{50} values, of this heterogeneous assay is higher by a factor of 30 than that obtained in homogeneous radioactive and non-radioactive assays with benzodiazepine receptors [18]. This gain in sensitivity may to some extent be explained by the low receptor concentration present, because there is no depletion of analyte by its receptor binding. Another factor is the

heterogeneous set-up of the assay. Competition for receptor binding between the analyte and the immobilized ligand only occurs in the diffusion layer of the wells in the microtiter plate. Over 99% of the receptor-containing proteoliposomes were bound to the immobilized ligand in the absence of analyte. This indicates that there is excess immobilized ligand relative to the amount of receptors and eventually also a high affinity. Because quantitative data concerning the amount of immobilized ligand per well were not available, no affinity for the immobilized ligand could be calculated.

Because low amounts of drugs can be detected, interference of biological matrices (e.g. plasma or urine) which frequently cause problems in the conventional receptor assay, are less of a problem in this novel receptor assay. Where normally a sample pretreatment step, such as solid phase extraction, would be needed to eliminate the presence of these interferences, a dilution of the biological sample could be enough to minimize the unwanted matrix effects in the receptor assay described in this thesis.
The procedure may be further improved by reducing the nonspecific binding; for example, using a further purified receptor preparation, selection of other microtiter plate materials, coating of the plate surface, addition of competing reagents in the incubation mixture, washing procedures or combinations thereof.

In conclusion, the fluorescent receptor assay described in this thesis appears to be a promising alternative for the conventional radioreceptor assay, as well as for nonradioactive receptor assays in which ligands are labeled by coupling with an easily measurable (e.g. fluorescent) functionality. Yet, additional studies are needed to further optimize the assay and, to perform comprehensive validations.

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Summary

Receptor proteins play an essential role in life. All organisms, from bacteria to plants, animals and human beings use receptors for their response to (external) signals. By definition, a receptor is a (macro) molecule which is able to *recognize* a distinct chemical entity (e.g. a hormone or neurotransmitter) amongst many others, and which *transduces* the information contained within that entity to the cell, resulting eventually in a response such as activation of an enzyme, alteration of membrane permeability etc. Most receptors are transmembrane glycoproteins naturally occurring in or on the cell membranes of different tissues and organs, although there are also soluble receptors found in the cytoplasm of the cell, e.g. receptors for steroids and thyroid hormones.
Besides endogenous compounds, poisons and drugs might also interact with a certain receptor; thereby mimicking or antagonizing the effects normally triggered by these endogenous molecules. The binding affinity depends on the potency of the drug. Thus, drugs with a high binding affinity can be administered in low dosages. The pharmaceutical industry, therefore, is constantly searching for new drugs with higher potencies, to keep the dosage of the drug as low as possible in order to minimize the total burden for the body and eventual side effects.

Ligand binding assays, including immunoassays and receptor assays, take advantage of the highly selective interaction between a binding protein (an antibody in the case of immunoassays or a receptor when receptor assays are used) and a ligand. Although immunoassays are widely used and are very sensitive in the quantitation of a variety of ligands, receptor assays can be used not only to determine the concentration of a ligand, but also its affinity for the

receptor. Besides, the raising of antibodies is a costly and laborious process, whereas receptors can be isolated in large amounts from animal tissue, obtained e.g. from the slaughterhouse, without much effort. The principle of the receptor assay is that, under well-controlled conditions, the receptors are incubated *in vitro* with a certain amount of labeled ligand and a (unknown) amount of analyte, which has a similar affinity for the same receptor. The amount of labeled ligand ultimately bound to the receptor depends on the concentration of analyte present in the sample. Thus, a low concentration of analyte will result in a high occupancy of the receptors by the labeled ligand, whereas at a high concentration of analyte only a small fraction of the labeled ligand will be bound to the receptors.

Two types of receptor assays can be distinguished:

1. **Saturation binding experiments**, in which constant amounts of receptors are incubated with increasing concentrations of labeled ligand. In this type of experiment the receptor binding sites will be occupied by the labeled ligand until, at a high concentration of labeled ligand, all receptors contain a bound ligand and, thus, are saturated. From these experiments the affinity (K_d) of the ligand and the maximum amount of binding sites (B_{max}) can be determined.
2. **Inhibition binding experiments**, in which constant amounts of receptors are incubated with a constant amount of labeled ligand and varying amounts of analyte. The (unlabeled) analyte will compete with the labeled ligand for the same receptor binding site. Increasing the concentration of analyte will result in lowering of the amount of labeled ligand bound to the receptor. Besides the K_d, also the IC_{50} (the concentration of analyte capable of displacing 50% of the maximum amount of bound labeled ligand) can be determined from these experiments.

Because of the low amounts of ligand and/or analyte (working levels typically in the n*M* - p*M* range), a label exhibiting a strong analytical signal is needed for sensitive detection of the bound ligand. So far, radioactive labels were used on many occasions, because of the high sensitivity and relative easy in obtaining these labels. The use of radioactivity, however, has several obvious disadvantages. Therefore, several attempts have been made recently in which fluorescent-labeled ligands were constructed. However, coupling of a large fluorescent group can easily result in a reduction of the affinity of the fluorescent-labeled ligand for its receptor, due to steric hindrance. Moreover, many positions on the ligand might be involved in the actual receptor binding and can therefore not be used for the coupling with the fluorescent moiety. The selection of a suitable ligand, an adequate fluorescent moiety and the development of the coupling procedure, is not an easy task and usually a process of trial and error.

This thesis describes the development of a novel fluorescent receptor assay for benzodiazepines. Instead of using a fluorescent-labeled ligand, the receptors were incorporated into fluorescent liposomes. To accomplish this, the receptor was solubilized from calf brain membranes and reconstituted in liposomes made of crude soybean lipids and fluorescent lipids. Furthermore, a ligand was immobilized to a microtiter plate to which the proteoliposomes containing the benzodiazepine receptors could bind. After washing the plate with ice-cold buffer, the bound proteoliposomes could be detected in the microtiter plate using a fluorescence plate reader. When analyte was added during the incubation, part of the receptors were displaced from the immobilized ligand resulting in a lower fluorescence signal after the washing step. Since many fluorescent lipids were present per single receptor protein, resulting in an amplification of the fluorescence signal, a gain in sensitivity was achieved, as

compared to classical fluorescent-ligand assays, in which only one label per receptor-ligand interaction is present.
The benzodiazepine receptor was selected as a model system, because of the experience present in our research group with the development of a radioreceptor assay for benzodiazepines and, recently, the development of a non-radioactive receptor assay for benzodiazepines, utilizing a fluorescent ligand. Besides, benzodiazepines are widely prescribed by physicians. A sensitive assay method is therefore necessary, e.g. for the screening of new drugs, for the testing of drugs of abuse and in clinical and forensic toxicology.

In **Chapter 1** the principle and theory of receptor assays is explained and a comparison with immunoassays is made. Methodological aspects, such as the selection and preparation of receptor material, the choice of the labeled ligand (radiolabels versus fluorescent labeled ligands) and methods for the separation of bound and free fractions of the ligand are discussed. Finally, different aspects of the solubilization of receptors (and membrane proteins in general) and the reconstitution of these receptors into lipid vesicles are described.

Chapter 2 describes the solubilization of the benzodiazepine receptor. To a crude calf brain homogenate, a suitable detergent was added. Sodium deoxycholate was chosen as the detergent and factors such as the concentration of detergent, de pH of the buffer and the ionic strength of the buffer, more specifically the addition of KCl, were optimized. To minimize the negative effects caused by the detergent, such as irreversible denaturation of the membrane proteins, the detergent concentration should be kept as low as possible. Although the binding characteristics of the solubilized receptors for the radiolabel were diminished compared to the membrane homogenate, as shown by the affinity constant K_d of 2.7 n*M* and a B_{max} of 0.40 pmol/mg protein for the solubilized preparation compared to a K_d of 1.46 n*M* and a B_{max}

of 0.85 pmol/mg protein for the membrane-bound receptors, the solubilized receptor suspension was found to be a good starting point for further experiments.

A better environment for receptors than just aqueous solubilization is a biological membrane, in which lipids surround, and stabilize, the membrane proteins. To mimic this situation, the solubilized benzodiazepine receptors were reconstituted into liposomes as described in **Chapter 3**. Solubilized lipids were mixed with the solubilized receptors and the detergent was removed by size-exclusion chromatography (gel-filtration), after which the proteoliposomes were formed. The mean size of the formed proteoliposomes was found to be 75 nm. To determine to what extent the choice of the lipid influences the binding characteristics of the reconstituted benzodiazepine receptor, several types of lipids, and mixtures thereof, were used in the reconstitution experiments. Asolectin, a crude soybean extract containing a mixture of several lipids, was found to be most suitable. The K_d was similar to that of the solubilized receptor, but the B_{max} was lowered, suggesting a loss in total binding sites or inactivation of part of the receptors. Yet, freezing and thawing led to an increase in the B_{max} values. The mean size of the proteoliposomes was doubled to 150 nm after the freezing and thawing cycle. The enlargement of the proteoliposome particle size might cause this increase in the B_{max}, with the curvature of the membrane playing a role in the functioning of the membrane proteins. Alternatively, the receptors were better inserted in the membrane during the freeze/thawing cycle, leading to activation of previously inactive receptor proteins.

The long-term stability of the proteoliposomes was assessed through storage of the proteoliposome suspension during a 2 month period at 4°C, –20°C or –80°C. Though storage at 4°C led to inactivation of the reconstituted

benzodiazepine receptor within one week, full activity of the receptors was maintained when stored at −20°C or −80°C.

Besides asolectin, fluorescent lipids could be co-incorporated in order to obtain fluorescent-labeled liposomes or proteoliposomes, as shown in **Chapter 4**. Three types of fluorescent lipids were used for these experiments, and fluorescein-DHPE was found to be suitable for strong labeling of the (proteo)liposomes. At a concentration of 250 µg fluorescein-DHPE per 150 mg total lipids, a 100,000-fold dilution of the liposomes suspension could be used in which the fluorescence signal was still significantly above background values. This is important since low amounts of proteoliposomes should be detectable when they are used in a fluorescent receptor assay.

Chapter 5 describes the immobilization of a suitable benzodiazepine ligand (didesethylflurazepam) to a microtiter plate. A modified radioreceptor assay showed that the principle of using proteoliposomes containing the benzodiazepine receptor in a microtiter plate containing immobilized ligand was valid. Because the binding between the receptor and the immobilized ligand takes place in a very small volume, near the walls of the plate, an increase in the sensitivity of the assay was observed.

In **Chapter 6** the development of the novel fluorescence receptor assay in microtiter plates is described. The fluorescent proteoliposomes, containing the benzodiazepine receptor, were added to the microtiter plate with the immobilized ligand. Addition of samples with increasing concentrations of analyte (lorazepam) led to displacement of the receptors from the immobilized ligand, in such a way that a clear-cut sigmoid relation was found between receptors found in the solution and the amount of analyte added. A factor 30 in sensitivity was gained using this assay compared to the traditional

radioreceptor assay. It can be expected that even higher sensitivities will be achieved when the assay is further optimized and validated.

Chapter 7, finally, gives some general conclusions about the work presented in this thesis, and gives recommendations for future experiments. Since protocols for the solubilization and reconstitution of many other types of receptors are available, and methods for immobilization of ligands for these receptors to solid phases are also described in the literature, it is expected that the novel fluorescent receptor assay approach as developed in this thesis will be generally applicable for other classes of pharmacologically active substances.

Samenvatting

Receptoren vervullen een belangrijke taak in alle hogere levende organismen. Deze eiwitten zijn of te vinden in het omhulsel van een cel, het zogenaamde celmembraan, of ze zijn aanwezig in het binnenste (vloeibare deel) van de cel, het cytoplasma. Receptoren hebben de eigenschap dat ze uit een grote hoeveelheid verschillende moleculen één, voor de receptor specifiek molecuul, of een groep van zeer verwante moleculen kunnen herkennen en binden, waarna dit een bepaalde reactie in de cel tot gevolg heeft. Bij mensen worden velerlei processen in het lichaam op deze wijze door receptoren gecontroleerd, zoals de bloeddrukregeling, de hormoonhuishouding en de signaaloverdrachten in de hersenen. Echter, lichaamsvreemde stoffen zoals vergiften en geneesmiddelen kunnen ook een binding aangaan met een bepaalde receptor, mits het vergift of geneesmiddel sterk lijkt op het lichaamseigen (endogeen) molecuul wat normaliter aan deze receptor bindt. Zoals verwacht kan worden maakt de farmaceutische industrie dankbaar gebruik van deze kennis. Immers, wanneer men weet wat de functie van een bepaalde receptor is, (bijvoorbeeld bloeddrukregulatie) en de structuur van het endogene molecuul is tevens bekend, dan kan een geneesmiddel worden ontworpen, die (zeer) sterk aan het receptor eiwit bindt, waardoor het gewenst effect bereikt wordt (zoals bloeddrukverlaging). Om precies te zijn, geneesmiddelen kunnen zó worden ontworpen, dat ze het normale effect van de receptoren kunnen imiteren dan wel verhinderen.

De binding, uitgedrukt in de bindingsaffiniteit, is een maat voor de sterkte van het geneesmiddel. Dit wil zeggen dat geneesmiddelen die een hoge bindingsaffiniteit bezitten in lage doseringen kunnen worden toegediend. Het spreekt dus vanzelf dat een zo hoog mogelijke affiniteit van een geneesmiddel

voor een specifieke receptor gunstig is, aangezien de dosering dan laag kan blijven, waardoor de totale belasting voor het lichaam minimaal is.

De receptoren die benodigd zijn voor het experimenteel werk, kunnen vrij eenvoudig verkregen worden uit dierlijk materiaal, hetzij slachtafval dan wel proefdieren. In de reageerbuis (*in vitro*) vindt de binding plaats tussen een bepaalde hoeveelheid receptoren en de chemische verbinding (ook wel ligand genoemd) hetgeen, na scheiding van het receptor-gebonden en vrije deel van het ligand, gemeten kan worden. Deze zogenaamde receptor assays zijn uniek als analyse methode, aangezien ze zowel de hoeveelheid van een bepaalde verbindingen kunnen bepalen, maar ook de mate van binding; de affiniteit. Twee soorten receptorassays kunnen worden onderscheiden:

1. **Verzadigingsexperimenten**, waarbij, in afzonderlijke reageerbuizen, aan een constante hoeveelheid receptoren een steeds hogere concentratie gelabelde (eenvoudig te detecteren) ligand wordt toegevoegd. Bij de hoogste concentraties ligand zal er verzadiging van de receptoren te zien zijn, hetgeen wil zeggen dat alle bindingsplaatsen bezet zijn met ligand. Uit deze experimenten kunnen zowel de bindingsaffiniteit van het ligand (K_d) en de maximale hoeveelheid bindingsplaatsen (B_{max}) bepaald worden.
2. **Verdringingsexperimenten**, waarbij een constante hoeveelheid receptoren wordt geïncubeerd met een constante hoeveelheid gelabeld ligand en een hoeveelheid ongelabeld ligand (analiet). Wanneer de concentratie van het analiet toeneemt zal het uiteindelijk het gelabelde ligand verdringen. Met andere woorden, bij lage concentraties analiet zal het grootste deel van het gelabelde ligand aan de receptoren gebonden zijn (sterk detectiesignaal) terwijl bij een hoge concentratie analiet de hoeveelheid gebonden, gelabelde ligand minimaal is (zwak detectiesignaal). Uit deze experimenten kan ook de K_d bepaald worden, maar ook een andere veel gebruikte

parameter: de IC_{50}, de hoeveelheid analiet die in staat is 50% van de maximale hoeveelheid van het ligand kan verdringen.

Om de binding tussen ligand en receptor te kunnen meten moet het ligand een geschikt label bevatten dat zeer gevoelig gemeten kan worden. Tot dusverre is er meestal gebruik gemaakt van radioactief gelabelde liganden, aangezien radioactiviteit relatief eenvoudig, nauwkeurig en zeer gevoelig te meten valt. Er kleven echter diverse nadelen aan het gebruik van radioactiviteit, waardoor de vraag naar niet-radioactieve detectiemethoden steeds groter wordt.
Een goed alternatief voor radioactiviteit is fluorescentie. Fluorescent-gelabelde liganden kunnen echter een (sterk) verminderde affiniteit hebben voor de receptor, aangezien een vrij grote fluorescente groep aan het ligand moet worden gekoppeld, hetgeen de receptor binding kan (ver)hinderen.

Dit proefschrift beschrijft de ontwikkeling van een nieuw type fluorescente receptor assay voor benzodiazepines. Het labelen van een ligand met een fluorescente groep werd vermeden door de receptoren in te bouwen in fluorescente liposomen (vetzuurbolletjes). Hiervoor was het noodzakelijk de receptoren (en een deel van de overige aanwezige eiwitten) te isoleren uit het membraan, waarna geschikte vetzuren werden toegevoegd en de (fluorescent-gelabelde) liposomen werden gemaakt. Het ligand werd vervolgens aan een geschikt dragermateriaal gekoppeld. Dit zogenaamde geïmmobiliseerde ligand was in staat de liposomen met receptoren te binden. Na een simpele wasstap, om de vrije fractie gelabelde liposomen van de gebonden fractie te scheiden, kon de gebonden hoeveelheid fluorescentie direct in het dragermateriaal gemeten worden. Een oplopende concentratie benzodiazepine in het monster was in staat competitie aan te gaan met het geïmmobiliseerde ligand, waardoor laatstgenoemde verdrongen werd van de receptor bindingsplaats. Met andere woorden, een hoge concentratie benzodiazepines in het monster resulteerde in

een lage hoeveelheid fluorescentie dat uiteindelijk op het dragermateriaal werd gemeten, terwijl een lage concentratie benzodiazepines in het monster een sterk fluorescentie signaal te zien gaven.
Wanneer gelabelde liganden worden gebruikt, is er per ligand-receptor binding 1 detecteerbare groep aanwezig. In de door ons ontwikkelde methode kunnen vele fluorescente vetzuren in hetzelfde liposoom ingebouwd worden, waardoor per ligand-receptor binding een groot aantal detecteerbare groepen aanwezig zijn. Dit resulteert in een versterking van het fluorescentie-signaal waardoor de gevoeligheid van de nieuw ontwikkelde receptor assay groter is dan bestaande assays.
De benzodiazepine receptor is als model gekozen, vanwege de ervaringen die onze onderzoeksgroep in de afgelopen jaren heeft opgedaan met zowel de ontwikkeling van een radioreceptor assay (dus gebruik makend van een radioactief isotoop voor detectie) voor benzodiazepines, als ook de ontwikkeling van een niet-radioactieve receptor assay voor benzodiazepines, waarbij een fluorescente groep aan het ligand werd gekoppeld. Bovendien zijn benzodiazepines (bijvoorbeeld Valium®) een belangrijke groep geneesmiddelen, aangezien ze zeer veelvuldig door de arts worden voorgeschreven. Een gevoelige assay methode is derhalve gewenst, niet alleen om de geschikte dosis te kunnen instellen (therapeutic drug monitoring) en voor screening van nieuwe geneesmiddelen, maar ook in de klinische en forensische toxicologie en bij het onderzoek naar geneesmiddelenmisbruik (o.m. drug abuse testing, verkeerstoxicologie, workplace testing).

Hoofdstuk 1 geeft een inleiding over het principe van receptor assays, waarbij tevens wat dieper wordt ingegaan op de theorie. Ook is er een vergelijking gemaakt met een ander soort ligand-binding assay, de immunoassay. Methodologische aspecten, waaronder de selectie en bewerking van receptor materiaal, de keuze van het gelabelde ligand (radiolabels en fluorescent-

gelabelde liganden) en de keuzemogelijkheden om de vrije en gebonden fractie ligand te kunnen scheiden worden hier eveneens besproken. Daarnaast wordt er aandacht besteed aan de mogelijkheden voor solubilisatie (oplosbaar maken) van membraaneiwitten en de reconstitutie (inbouwen in liposomen) van de receptoren.

In **Hoofdstuk 2** wordt de solubilisatie van de benzodiazepine receptor beschreven. De receptoren, die in grote membraanfragmenten zitten, werden met behulp van een detergens van de lipiden (vetzuren) gescheiden. Natriumdeoxycholaat werd gekozen als detergens, en factoren zoals de concentratie detergens, de pH van de buffer en de ionensterkte (toevoeging van KCl) werden geoptimaliseerd. Hoewel de bindingseigenschappen van de gesolubiliseerde receptoren voor het radiolabel [^{3}H]flunitrazepam ([^{3}H]FNM) wel veranderen (een affiniteitsconstante (K_d) van 2.7 n*M* en een maximale hoeveelheid bindingsplaatsen (B_{max}) van 0.40 pmol/mg eiwit, vergeleken met een K_d van 1.46 n*M* en een B_{max} van 0.85 pmol/mg eiwit voor membraan-gebonden receptoren) is deze gesolubiliseerde receptorsuspensie zeer zeker geschikt als uitgangsmateriaal voor verdere experimenten.
Aangezien de toevoeging van detergentia aan membranen een negatief effect kan hebben op de werking van de membraaneiwitten dient de concentratie van het detergens zo laag mogelijk te worden gehouden.

In **Hoofdstuk 3** wordt de reconstitutie van gesolubiliseerde receptoren in liposomen beschreven. Gesolubiliseerde lipiden werden gemengd met de gesolubiliseerde receptoren en, via gel-filtratie, werden proteoliposomen (liposomen met eiwitten) gevormd. De gemiddelde grootte van de gevormde proteoliposomen was 75 nm. Onderzocht werd welke lipiden van belang zijn voor behoud van de bindingseigenschappen van de benzodiazepine receptor. Een mengsel van lipiden (asolectine, een extract van lipiden van de soja boon),

bleek de bindingseigenschappen het minst te verstoren. De K_d bleef vrijwel onveranderd, maar de B_{max} nam wel verder af, hetgeen wijst op verlies van de totale hoeveelheid bindingsplaatsen, dan wel inactivatie van een deel van de receptoren. Na een cyclus van vriezen (–70°C) en ontdooien (+20°C) werd er een stijging in de B_{max} waargenomen. Hoewel de gemiddelde grootte van de liposomen met een factor twee toeneemt tot 150 nm, waardoor het grote receptor complex wellicht beter kan functioneren, kan het ook zo zijn dat de receptor gedurende de vries/dooi cyclus beter de gelegenheid krijgt zich goed in het membraan te positioneren. Een deel van de receptoren die voor de vries/dooi cyclus inactief was, werd derhalve actief na deze cyclus.

Om de stabiliteit van de gereconstitueerde receptorsuspensie vast te stellen werd deze voor een langere periode bewaard bij 4°C, –20°C en –80°C. Hoewel de receptoren die bij 4°C bewaard werden al na een week vrijwel inactief waren, bleek opslag bij –20°C en –80°C nauwelijks van invloed op de activiteit gedurende twee maanden.

Een betere omgeving voor de receptoren is een membraan, waarbij lipiden de receptoreiwitten omhullen en stabiliseren.

Door naast asolectine ook fluorescente lipiden toe te voegen aan de gesolubiliseerde receptoren, worden na reconstitutie fluorescent gelabelde proteoliposomen gevormd, hetgeen beschreven wordt in **Hoofdstuk 4**. Drie verschillende fluorescente lipiden zijn gebruikt tijdens de experimenten, waarbij is gebleken dat fluoresceine-DHPE geschikt is om de liposomen sterk te labelen. Bij een concentratie van 250 µg fluoresceine-DHPE per 150 mg lipiden kon de proteoliposoom suspensie 100.000 keer verdund worden, waarbij het fluorescentie signaal nog steeds meetbaar was. Dit is belangrijk aangezien er in de te ontwikkelen receptor assay in een aantal gevallen (waarbij de concentratie analiet in het monster hoog is) slechts een gering aantal gelabelde proteoliposomen moet kunnen worden gemeten.

In **Hoofdstuk 5** wordt de koppeling van een geschikte benzodiazepine (didesethylflurazepam) aan een microtiterplaat beschreven, waarna met een aangepaste radioreceptor assay het principe werd aangetoond dat proteoliposomen (met de benzodiazepine receptor) aan geïmmobiliseerde liganden kunnen binden. Doordat de binding tussen geïmmobiliseerd ligand en de receptor slechts in een zeer klein gebied plaats heeft (namelijk de wanden van de microtiterplaat), werd een verbetering van de gevoeligheid van de assay waargenomen.

In **Hoofdstuk 6** wordt de ontwikkeling van de nieuwe fluorescente receptor assay in microtiterplaten beschreven. De fluorescente proteoliposomen, die de benzodiazepine receptor bevatten, werden toegevoegd aan de microtiterplaten met geïmmobiliseerd ligand. Toevoeging van monsters met oplopende concentraties (ongelabelde) analiet resulteerde in verdringing van de proteoliposomen van geïmmobiliseerd ligand. Een factor 30 winst in gevoeligheid vergeleken met traditionele radioreceptor assays werd gevonden. Verwacht wordt, dat wanneer de assay verder geoptimaliseerd en gevalideerd is, een nog hogere gevoeligheid bereikt kan worden.

Hoofdstuk 7, tenslotte, geeft de algemene conclusies over de in dit proefschrift beschreven werk, en geeft enkele aanbevelingen voor toekomstige experimenten. Aangezien de koppeling van liganden aan dragermaterialen en solubilisatie- en reconstitutie protocollen voor diverse andere receptoren in de literatuur zijn beschreven, is de verwachting gerechtvaardigd dat een soortgelijke fluorescente receptor assay als hier beschreven voor de benzodiazepine receptor, ook toegepast kan worden voor andere receptoren.

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